CHARACTERIZATION OF THE FUNCTION OF VACCINIA VIRUS GENES A18R AND J3R IN TRANSCRIPTIONAL REGULATION OF POSTREPLICATIVE GENES

By

Ying Xiang

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I dedicated my dissertation to my husband, Jiong Shi, for his care, love and support, and to my grandmother and my parents who taught me about love.

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Ying Xiang

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Chairman: Richard C. Condit, Ph.D.
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Gene expression in prokaryotes and eukaryotes is controlled primarily by the regulation of transcription. Transcription initiation is well defined to some extent in both systems. Transcription elongation and termination of eukaryotes is not well defined though tremendous efforts have been put into this research. Vaccinia virus replicates in the cytoplasm of the infected host thus requiring that the virus encode the machinery necessary for both viral RNA and DNA metabolism. Therefore, it presents a good model system to study transcriptional regulation. Transcription termination of early viral genes is controlled by the viral RNA polymerase, the capping enzyme and NPH-1 in response to a cis-acting sequence in the nascent RNA transcripts. However, the mechanism of transcription termination of postreplicative genes is not yet known. In this dissertation, the function of a viral gene product, A18R, and its interaction with G2R and J3R in

transcriptional regulation are investigated. Our previous *in vivo* characterization of A18R mutant viruses shows a "promiscuous transcription" phenotype at late times postinfection under nonpermissive conditions, which is transcription in the regions of the genome that are normally transcriptionally silent. Transcriptional analysis of a region which contains three early genes (M1L, M2L and K1L) positioned directly downstream of the intermediate gene, K2L, indicates that mutation of the A18R gene results in increased amounts of readthrough transcription of the M1L gene originating from the K2L intermediate promoter. This implies that the A18R gene product is a negative transcription elongation factor or a termination factor for postreplicative genes. Our previous *in vivo* characterization of a G2R mutant suggests that G2R positively regulates transcription elongation of postreplicative genes. The G2R mutant viruses produce late transcripts that are truncated at the 3' ends. An interaction between A18R and G2R has been demonstrated by genetic analysis. Specifically, a double mutant virus containing both A18R and G2R mutations is viable, and the phenotypic revertants of A18R have been mapped to the G2R gene and the J3R gene. J3R mutant viruses display a defective late phenotype similar to the G2R mutant viruses. The J3R gene product is both a poly (A) polymerase subunit and an mRNA nucleoside-2'-O-methyltransferase. *In vitro* biochemical analysis shows that an A18R suppressing mutation in J3R gene abolishes the ribose methylation function. Both *in vitro* and *in vivo* analyses suggest that the poly (A) formation in the J3R mutant virus is not affected. In summary, we believe that the A18R, J3R and G2R gene products interact with each other either directly or indirectly as a part

of a higher order complex to regulate transcription elongation and termination of postreplicative genes and mRNA metabolism.

CHAPTER 1
INTRODUCTION

Transcription Regulation in Prokaryotic and Eukaryotic Systems

Transcription in both prokaryotes and eukaryotes involves synthesis of an RNA chain representing one strand of a DNA duplex. RNA synthesis is catalyzed by RNA polymerase and is controlled by trans-acting factors in concert with the RNA polymerase. The nascent eukaryotic mRNA is then modified posttranscriptionally by 5' capping and 3' polyadenylation. In contrast, prokaryotic mRNA is not modified. Transcription can be divided into several distinct processes that include promoter binding, RNA chain initiation, elongation and termination. Regulation of transcription can occur at any of these steps. Transcription initiation in eukaryotes has been well studied during the last 15 years and substantial progress has been achieved, while the mechanism of transcription elongation and termination are not yet well understood. Given the complexity in studying eukaryotic transcription, vaccinia provides a good model system because vaccinia virus encodes virtually all of the machinery required for mRNA synthesis and its gene synthesis is very similar to that of eukaryotes. For example, vaccinia virus coded capping enzymes and poly (A) polymerase have provided excellent models for 5' capping and 3' polyadenylation in eukaryotes. Transcription initiation of three classes of vaccinia

and transcription termination of early stage genes have been investigated, however, little is known about transcription elongation and termination of intermediate and late genes. The goal of this dissertation is to study the regulation of transcription elongation, termination and posttranscriptional modification of postreplicative genes in vaccinia virus. We hope that this study will have some impact on understanding transcription elongation/termination in eukaryotic systems. We report here that vaccinia gene A18R encodes a negative transcription elongation factor for postreplicative genes and is involved in a higher order complex containing RNA polymerase, a positive transcription elongation factor (the G2R gene product), and a 2'-O-methyltransferase and polyadenylation stimulatory factor (the J3R gene product). Before presenting the evidence, I would like to describe RNA polymerase and transcriptional regulation in prokaryotes and eukaryotes.

RNA Polymerase

Transcription in both prokaryotes and eukaryotes is catalyzed by a multisubunit RNA polymerase with different compositions. The 480 kD bacterial RNA polymerase core enzyme (subunit structure: $\alpha_2\beta\beta'$) contains the catalytic machinery for RNA synthesis. The 160 kD β' subunit is involved in binding the template DNA and the 150 kD β subunit is involved in binding the ribonucleotide substrate. Both the β' and β subunits can be crosslinked to DNA. The 40 kD α subunit is required for assembly of the core enzyme with no direct effect on transcription. The 550 kD eukaryotic RNA

polymerase contains 12 subunits, two large subunits and a collection of small components (258). The small polypeptides have sizes ranging from 10 to 40 kD and vary in number from 6 to 10. The largest subunit is about 200 kD in size. Its binding with the DNA template is demonstrated by blotting and UV cross-linking experiments. It may also bind with the nascent RNA chain. Its participation in chain elongation is further supported by the evidence that the mutants of the 200 kD protein from yeast and drosophila are defective in elongation *in vitro*. The second largest subunit is about 150 kD and is not phosphorylated. It is probably involved in binding the nucleotide substrate and phosphodiester bond formation (242). Therefore, there is strong biochemical, immunological and genetic evidence that the two large subunits of eukaryotic RNA pol II share functional homology to β and β' in the bacterial enzyme (8;280).

In contrast to bacterial RNA polymerase, the C-terminal domain (CTD) of the largest subunit of eukaryotic RNA polymerase has significance in transcription. It consists of tandem repeats of the consensus heptapeptide sequence YSPTSPS. The hydrophilic CTD is fully exposed to the solvent at the surface of the enzyme. It plays an essential role *in vivo* because deletion of most or all of the CTD is lethal to cells (211). CTD is also important in efficient basal and activated transcription initiation *in vitro* (157). Furthermore, CTD phosphorylation plays a role in the transition from transcription initiation to elongation (160;216).

Although both prokaryotic and eukaryotic RNA polymerase core enzymes contain the catalytic function, they cannot initiate transcription at the proper sites. The RNA

polymerase needs to interact with transacting factors to carry out specific transcription initiation, elongation and termination.

Transcription Initiation

The process of transcription initiation consists of promoter binding, preinitiation complex assembly, preinitiation complex activation (DNA melting), RNA chain initiation and promoter escape. Transcriptional promoters contain sequences to be recognized by RNA polymerase or associated factors.

In prokaryotic systems, promoter elements have been identified through sequence comparison and mutagenesis studies. The conserved features of promoters have been determined by comparison of more than 100 promoters. The optimal promoter, which contains two conserved hexamers centered around -10 (TATAAT) and -35 (TTGACA), has been defined by mutagenesis. Bacterial RNA polymerase can bind to all the promoters with no specificity. The ability to bind stably and initiate specifically at particular promoters is controlled by sigma factors (44). Sigma factors are also involved in melting DNA to open the preinitiation complex and to form a transcription bubble.

In eukaryotic systems, the basal promoter element, the TATA box, is located approximately 30-nt upstream from the transcription start site. Promoter recognition and transcription initiation are mediated by a complex of transcription factors in addition to a RNA polymerase. The general initiation factors have been identified and purified, and working models for their roles in initiation have been established. TFIID is the only factor capable of sequence specific binding to a eukaryotic promoter element, and thus is

functionally similar to the prokaryotic sigma factors. TFIIF of TFIID binds the TATA box in the first step of forming a transcription initiation complex. TFIIB then binds to the polymerase. TFIIH contains both a helicase involved in DNA melting, and a protein kinase responsible for extensive phosphorylation of the CTD of polymerase II. CTD phosphorylation could serve to dissociate the complex which is bound to RNA polymerase II through the CTD and lead to the association of other proteins specific for elongation.

In some cases, the activity of the promoter is increased by the presence of an enhancer, which consists of another group of elements which are located at variable distances from those promoters. Enhancers can increase the basal level of transcription in a manner that is independent of their orientation and distance relative to the RNA start site. They can activate a gene in a specific cell type or at a particular stage in development, therefore, they play critical roles in controlling the differentiation and development of both prokaryotic and eukaryotic organisms and in regulating their metabolism. The precise mechanism of enhancer function is not known. It is thought to function by increasing the concentration of transcription factors in the vicinity of the promoter. An attractive "facilitated tracking" model to illustrate the interaction between the enhancer and the promoter was proposed recently (30). This model incorporates elements from both the DNA looping model and the DNA scanning model. An enhancer-bound complex has been proposed to "track" along the DNA template until it encounters the cognate promoter, at which a stable looped structure is formed. Since

vaccinia virus does not contain enhancer elements, I will focus on basal transcription in the dissertation.

Transcription Elongation

The point at which transcript elongation actually begins is not clearly defined. For *E.Coli*, the transition from the initiation phase to the elongation phase is characterized by three distinct biochemical changes in the RNA polymerase complex: the sigma factor is released, RNA polymerase is cleared from the promoter, and a tight ternary complex is formed containing RNA polymerase, the nascent transcript and the DNA template. For eukaryotes, the reaction is similar but is more complicated and less well defined. It involves the dissociation of numerous initiation factors (325) and phosphorylation of the C-terminal domain of the largest subunit of RNA polymerase (68).

During the transcription elongation phase, RNA polymerases catalyze successive polymerization of ribonucleotides into a transcript based upon their complementarity to bases on a DNA template. The elongation is processive but discontinuous. The rate of chain elongation varies among RNA polymerases and correlates with the subunit complexity of the enzyme (reviewed in 293). Bacterial RNA polymerase synthesizes RNA at 50-100 nt per sec *in vivo* and 10-35 nt per sec *in vitro*. Vaccinia RNA polymerase synthesizes RNA at an average rate of 17 nt per sec *in vitro*. Eukaryotic RNA pol II elongates at 20-30 nt per sec *in vivo*. The transcription rate is due in part to the tendency of the enzyme to pause at certain locations.

During elongation, RNA polymerase may stop transcription without releasing the transcript, a process referred as a pause or transcription arrest. During a transcription pause, RNA polymerase temporarily stops RNA synthesis for a period of time before resuming transcript elongation. The 3' end of the nascent transcript remains within the catalytic site of a paused RNA polymerase. In contrast, the 3' end of the nascent transcript is displaced from the catalytic site of an arrested RNA polymerase. To resume transcription elongation from an arrested complex, the transcript must be cleaved to remove 2-10 nucleotides from the 3' end of the transcript in order to return the 3' end to the catalytic site of the polymerase. The RNA polymerase itself catalyzes the cleavage, which is activated by trans-acting factors GreA (prokaryotes) and SII (eukaryotes) (219;249). Therefore, an arrested elongation complex is unable to efficiently resume transcription without the aid of accessory factors. Paused and arrested complexes cannot be distinguished *in vivo*.

Three speculative models of the elongation process have been proposed based on the study of the features of the "walking" and halted ternary elongation complexes (TEC). The monotonic model (296) hypothesized a rigid RNA polymerase conservatively advanced 1 nt along the template as one ribonucleotide was added to the 3'end of the nascent transcript until the RNA:DNA hybrid was partially disrupted by a hairpin formation. Alternatively, a very liberal inchworm model was proposed by Chamberlin (147) based on the differences of sizes of DNA and RNA footprints of halted transcription elongation complexes. In this model, RNA polymerase moved forward with alternative ends discontinuously like an inchworm and freely took more than one

ribonucleotide at a time. The polymerase maintained at least one tight contact with the template by alternatively locking and sliding of front and back DNA and RNA contacts, resulting in shrinking DNA footprints (33 bp to 25 bp) and growing RNA footprints (18 bp to 25 bp). Transcription was paused as a hairpin was formed or arrested when the active site was relocated backwards along the RNA. To make a conservative RNA polymerase do a liberal job came the third model--the sliding clamp model (142;215;233). Rather than being flexible, the RNA polymerase could be relatively rigid. Except for some specific sites, this enzyme could move along the DNA template monotonously (213;214). After halting, it could slide backward along the DNA and RNA shifting the RNA:DNA hybrid and the transcription bubble with it. The active center was preserved during transcription elongation and presumably maintained during backtracking. It was shown that 16 to 24 nt of nascent transcript was protected by the elongation complex and an 8 bp RNA:DNA hybrid positioned the RNA 3' end in the active site 1 to 3 nt from the point of DNA strand separation (215). It was demonstrated that the constant-sized RNA and DNA footprints moved upstream as a halted transcription complex arrested (141). Results from yeast polymerase II showed the similar transient backtracking phenomenon (233). It was predicted that the backtracking of the transcription complex was induced by an unstable 3' proximal RNA-DNA hybrid because the sequences behind and ahead of the hybrid were reported to affect arrest (147;213;214). In fact, the hybrid was shown to unwind during arrest. The rate of arrest decreased if the hybrid was strengthened by incorporation of nucleotide analogs to stabilize the hybrid, such as 5-bromoUTP and 5-iodoCMP. The rate of arrest increased

with the helix-destabilizing analog, such as thiouridine and inosine (215). Most importantly, destablization of the 3' proximal DNA:RNA hybrid lead to backtracking of the RNA polymerase shown by footprint analysis.

The molecular regulatory mechanism for the processes of pausing and arrest is not known. It has been shown that both intrinsic and extrinsic factors can block transcription elongation. The intrinsic factors include RNA secondary structures or specific RNA sequences. Results from several labs have shown that RNA secondary structure is implicated in the mechanism of the intrinsic transcriptional block in prokaryotes (10;147). In both prokaryotes and eukaryotes, a specific T-rich sequence in the nontranscribed strand can be recognized "intrinsically" by purified RNA polymerase II as a block to elongation (106;107;140;214). The specific sequence may cause the bending of the DNA strand, which is directly responsible for inducing arrest (140). In addition, RNA polymerase can be stopped by extrinsic sequence-specific proteins (140). There are numerous examples of viral, prokaryotic and eukaryotic RNA polymerases being physically blocked by DNA-binding proteins during elongation. These include lexA (252), lac repressor (76;236), Reb1p (150), and the CCAAT-box-binding protein (63;64). Numerous transcription elongation factors have been found to assist RNA polymerase through blocked sites. Mechanisms include association with the polymerase prior to an elongation block to increase the rate of transcription elongation or stimulation of an arrested polymerase to cleave the transcript to restore the 3' end of the nascent transcript to the catalytic site. For example, the eukaryote TFIIF and elongin/SIII complex can suppress transient pausing, while the prokaryotic GreA, GreB and the eukaryote SII can

stimulate the cleavage event by RNA polymerase. The prokaryotic and eukaryotic elongation factors are detailed below.

In prokaryotes, the transcript cleavage reaction first described by Chamberlin and coworkers (279) leads to cleavage and removal of 3' end oligonucleotide of nascent RNA in a halted elongation complex. Elongation continues from the newly generated 3' end of transcripts after cleavage if NTPs are present. GreA and GreB have been shown to mediate the RNA cleavage at the 3' end of nascent RNA and release transcription arrest of a paused transcription complex (33;234). The 158-amino acid GreA interacts directly with RNA polymerase and it must be present in the transcription complex before the polymerase reaches the arrest site. GreB is effective when it is added to a paused complex.

In eukaryotes, the elongation factor SII is a 38 kD protein which was discovered and purified from extracts of Ehrlich ascites tumor cells (257). SII has since been purified from a variety of eukaryotes. SII can increase the overall rate of RNA polymerase II elongation by helping the polymerase through a variety of transcriptional impediments rather than by increasing the catalytic rate of RNA polymerase. Considerable evidence has been found to support the notion that SII promotes cleavage of nascent transcripts in the RNA polymerase II elongation complex. First, SII-dependent elongation by RNA polymerase is accompanied by nascent transcript cleavage (127;193;235). Second, SII-dependent cleavage products appear prior to the readthrough products (127;235). Third, in human histone H3.3 gene, all SII-dependent transcripts extended beyond intrinsic arrest sites are cleaved (235). Fourth, SII deletion or point

mutants that fail to activate nascent transcript cleavage also fail to promote readthrough (51;235).

TFIIF is a heterodimer composed of 30 kD (RAP30) and 70 kD (RAP70) subunits. It is a unique transcription elongation factor because it controls RNA polymerase at both the initiation and elongation stages of transcription. It promotes transcription initiation in part by stabilizing binding of RNA polymerase II and TFIIB to the TFIID-promoter complex (11;55). It functions in transcription elongation by suppressing transient pausing to increase the overall catalytic rate of RNA polymerase II (228).

The elongin/SIII complex is composed of three subunits, elongin A, B and C, with molecular mass of 110, 18 and 15 kD respectively (35;36). The elongin A subunit can increase the overall rate of RNA pol II transcription by suppressing transient pausing (12). Elongin C can activate the elongin A and the specific activity of elongin AC complex is significantly increased compared to elongin A alone. Elongin B does not stimulate elongin A activity directly, but it functions as a molecular chaperon that facilitates the assembly of the elongin complex (284).

In the drosophila system, two classes of elongation complex have been identified (168). The first abortive RNA polymerase elongation complex is formed after transcription initiation at a promoter. The abortive elongation generates short transcripts due to the polymerase pausing caused by a negative transcription elongation factor N-TEF. N-TEF will be addressed in detail later. The second elongation complex carries out productive elongation in which long transcripts can be synthesized. The productive

elongation complex is derived from the early paused elongation complex by the action of a positive transcription elongation factor P-TEFb. P-TEFb was originally isolated from Drosophila extracts and then purified to homogeneity (169). It is a heterodimer composed of 124 kD and 43 kD polypeptides. It contains a protein kinase activity that can phosphorylate the CTD of RNA pol II and it controls the transition from abortive elongation into productive elongation (167).

Transcription Termination

Transcription termination is a process consisting of the recognition of an intrinsic termination signal in nascent transcripts by RNA polymerase and the cessation of RNA chain elongation, followed by release of the RNA polymerase from the DNA template (122). In prokaryotes, most steady state transcript 3' ends are formed directly by a termination event. A variety of studies have shown that at least two mechanisms control transcription termination. First, rho-independent termination is mediated by RNA polymerase in response to a terminator sequence. Among the multipartite terminators, a consensus sequence which contains stable RNA hairpin (3 to 5-nt loop) with a G+C rich stem (5 to 9-bp stem) and a uridine-rich sequence immediately after the hairpin has been identified. More recently, Chamberlin and coworkers, using a mutagenesis assay, have defined two additional components of termination sites, the DNA or RNA sequence upstream from the terminator hairpin and the DNA sequence downstream from the release site (237;238). The model for termination suggested that the stem-loop structures could promote pausing of RNA polymerase, the U-rich terminator could destabilize the

interaction of paused RNA polymerase with the template, and termination occurred near the end of the U-runs (310). Combined with the backtracking model, the hairpin structure was proposed to prevent backsliding and confined polymerase to unstable U-rich configuration. To make escape from the paused state and continue elongation unlikely, termination ensues. This speculation is based on the observation of the importance of the spacing between the hairpin and RNA 3' end during elongation (47;78). Second, rho-dependent transcription termination is caused by interaction of the rho protein with RNA polymerase that is paused at a downstream site of the nascent transcript. Termination occurs when the ternary elongation complex finally dissociates (240). The rho gene has been cloned and it encodes a 419 amino acid protein which is active as a hexamer (85). Rho binding sites are generally unstructured, C-rich and G-poor, and show little sequence conservation. Rho binds to these 80-nt regions to allow termination downstream. Rho is an RNA binding protein, belonging to the small nuclear ribonucleoprotein particle (snRNAP) family. Rho also displays RNA-dependent ATPase and helicase activities, which it is thought serve to translocate the enzyme along the RNA in an ATP-dependent manner. Both RNA binding and ATP hydrolysis are required for Rho activity. The interaction of Rho with RNA polymerase is influenced by additional factors of the elongation complex. Rho has been proposed to act on RNA polymerase via an interaction with the NusG protein (156;277). Gottesman and co-workers discovered that cells depleted of NusG are defective in Rho-dependent termination. Recent *in vitro* studies reveal that the presence of NusG allows Rho to cause termination at several termination sites more efficiently (45;203)

At some terminators, the termination event can be prevented by specific ancillary factors that interact with RNA polymerase. Antitermination factors cause the RNA polymerase to continue transcription past the terminator sequence (reviewed in reference 102). The antitermination phenomenon was discovered with phage lambda. Both the lambda N and lambda Q proteins can participate in antitermination at a variety of terminators, which are rho-dependent or rho-independent. Lambda N protein suppresses termination by binding to the RNA transcript upstream of the terminator at a boxB hairpin structure (the N utilization or Nut site). In contrast, the lambda Q protein binds to the promoter initiation complex and suppresses polymerase pausing as well as increasing the number of transcripts that read through the termination sequence. The mechanism by which Q protein functions has been partially elucidated (320). It has been proposed that Q protein is added to the elongation complex with the facilitation of another elongation factor, Nus A, at the site where RNA polymerase paused. Then the Q-modified RNA polymerase is able to read through downstream pause sites and termination sites. For N protein-mediated antitermination, at least four additional bacterial proteins (NusA, NusB, NusG and S10) are required and may proceed by a mechanism similar to but more complicated than lambda Q anti-termination (187). Most of these accessory factors may require N activity as suggested by genetics and biochemical assays (153;278). The lambda N protein interacts with RNA polymerase along with these four proteins, assembling on the boxB RNA of the nut site to form a functional antitermination complex (Fig. 1-1). Specifically, RNA polymerase is bound by NusA, S10, and NusG. N protein binds to NusA and also to the RNA hairpin boxB. NusB forms a dimer with S10 that

binds boxA. It is thought that this complex forms as soon as the Nut site is transcribed and then persists through downstream RNA synthesis (209). For terminators close to the promoter, the subset of elements N, NusA, and boxB suffices for antitermination *in vitro*, whereas more distant terminators require the full set of factors (175). Presumably the additional factors stabilize the antitermination complex and allow the extensive antitermination event to occur. Thus, upon recognizing the nut site, lambda N acts on RNA polymerase through protein-protein interactions to ensure that the enzyme can no longer respond to the terminator.

In eukaryotes, transcription termination is strikingly different from what occurs in prokaryotes. Almost all steady state transcript 3' ends are generated not by transcription termination, but rather by processing of the primary transcript. 3' processing will be addressed in detail later in this chapter. Transcription termination usually occurs at heterogeneous sites downstream of the polyadenylation signal of many mRNA genes (230). Experiments with several genes suggest a dependence of transcription termination on 3' end processing (62;81;151;159;230;309). Specifically, mutations interfering with polyadenylation inhibit transcription termination, and insertion of a polyadenylation site can induce termination. Therefore, the termination of transcription by RNA polymerase II may be dependent on the assembly of a functional 3' processing complex on the nascent RNA (300;302).

The discovery that active 3' processing is required for termination of transcription has led to different models for the mechanism of termination. One model proposes that a specific elongation factor enables the RNA polymerase to transcribe efficiently through a

gene, but this factor is released upon encountering a poly (A) signal. Without its elongation factor, the polymerase is more prone to random termination resulting in heterogeneous termination beyond the end of the gene. The second model proposes that as soon as RNA polymerase II passes a poly (A) site, 3' processing activities can potentially act on the nascent transcript to release the mRNA sequence. The polymerase may continue to transcribe downstream sequences and it may still attach to a growing nascent transcript with its 5' end corresponding to the 3' end of the mRNA sequence. This newly formed 5' terminus is unprotected by a 5' cap structure and must therefore be subject to nucleolytic degradation such as a 5' to 3' exonuclease activity. When the nascent transcript is completely degraded the pol II is released from the DNA effecting termination of transcription.

The authentic termination site of a eukaryotic gene is very difficult to identify. Primary transcripts are very short lived for two reasons, (i) RNA processing, including both splicing and 3' end processing can occur either during or immediately following RNA synthesis; (ii) nonspecific nucleases in the nucleus can rapidly degrade primary transcripts. The position of termination has been characterized for several pol II genes by nuclear run-off analysis, and is generally heterogeneous, occurring between 100 and 4000-nt downstream of the poly (A) site (52;109;230). The observed heterogeneity of termination downstream from poly (A) sites may be influenced by specific features of a gene. First, the efficiency of the poly (A) site may affect the site of termination. If a gene has an inefficient poly (A) site, the polymerase may have to travel a considerable distance past the poly (A) site before cleavage occurs. Second, sequences in the 3'

flanking region may affect its site of termination. In eukaryotes, a termination sequence could take the form of RNA secondary structure, unusual DNA structure, a protein-binding site, or a combination of these. It has been shown that a potential hairpin-loop structure in the 3' flanking region of the chicken β-globin gene is implicated in transcriptional termination (227). This hairpin structure may slow down or pause the RNA polymerase and may give helicase and exonuclease activities more time to catch up with the polymerase. Another reason for the stalling of RNA polymerase II might be the presence of a protein factor bound to the DNA template. A CCAAT box binding protein has been shown to cause termination in the adenovirus major late promoter (63;64). This factor could directly block the passage of the elongating RNA polymerase. The presence of several pause sites in the 3' flanking region of a gene could cause a fraction of elongating polymerases to pause at each site, resulting in heterogeneous termination.

Involvement of eukaryotic transcription factors that negatively regulate transcription and promote transcript release has been proposed in drosophila, frogs, mice and yeast systems. Both positive transcription elongation factors (P-TEF) and negative transcription elongation factors (N-TEF) exist and that they balance the transcription elongation and termination to make normal transcripts. To date, many P-TEFs are identified in human, murine, Drosophila, and Xenopus systems, which have been described above. However, limited N-TEFs are identified. Drosophila factor 2, a Drosophila N-TEF protein, causes abortive transcription elongation and induces transcript release in the absence of P-TEF (319). N-TEF is a double-stranded DNA dependent ATPase which lacks detectable helicase activity (318). It can cause the release

of transcripts by RNA polymerase II in an ATP-dependent manner (319). Yeast REB1 protein is another N-TEF which regulates transcription elongation and termination of RNA Pol I. The Pol I termination site is a sequence of approximately 61bp encompassing an 11 bp binding site for Reb1p and an essential 5' flanking sequence containing a release element. Binding of the REB1 protein to its binding site pauses the polymerase over the release element. Termination then occurs at 14-20 nt upstream of the REB1 binding site (150). TTF-1 binds to a specific 18 nt-long element called the Sal box and it is required for termination of mouse rRNA genes (105;116;177). The frog Rib2 protein, which binds to a GACTTGCNC site, is required for termination of transcription by RNA Pol I (178). In vaccinia, capping enzymes and NPH-1 mediate RNA polymerase termination 30-50 nt downstream of the specific sequence T_5NT (75). The details of vaccinia transcription termination will be discussed later. Thus it is clear that transcription termination in several systems requires the participation of factors which can bind single or double stranded RNA or DNA and hydrolyze ATP. The precise mechanism of action of these factors is not known, however, it seems reasonable that translocation or helicase activities of these factors within an elongation complex may destabilize the complex.

Posttranscriptional Modification of Eukaryotic and Viral mRNA

5' capping, polyadenylation and RNA splicing are significant modifications that occur only in eukaryotic cells and their viruses. Though there have been many reports

during the last 20 years documenting the presence of short (10 to at most 25-nt) 3' adenylates on a few percent of bacterial mRNAs, most investigators doubt that bacterial polyadenylation has any functional consequences. Since vaccinia capping enzymes and poly (A) polymerase have provided precedents for understanding eukaryotic and viral mRNA posttranscriptional modification, I will focus on vaccinia virus mRNA posttranscriptional modification and extend the discussion to eukaryotic and other viral posttranscriptional modification in this section. I will discuss the life cycle and transcription of vaccinia virus later.

5' Methylation

The 5' terminal cap is a ubiquitous feature of eukaryotic messenger RNA. The cap structures are classified on the basis of the number of methyl groups that are present. Caps with methylation on the N^7 position of guanosine alone are referred to as cap 0 (m^7GpppN). Cap 1 has 2'-O-methylation on the first transcribed nucleoside (m^7GpppN^m) and cap 2 has two 2'-O-methylations on the first and second transcribed nucleosides ($m^7GpppN_1^mpN_2^m$). All eukaryotic cellular mRNAs and most viral mRNAs, whether of animal, plant, or insect origin contain characteristic 5' cap structures. Exceptions include picornavirus (117;210) and satellite tobacco necrosis virus (311) RNAs. Cap 0 structures are found in tobacco mosaic virus and brome mosaic virus (70;138;328). Cap 1 structures are added to *in vitro* transcripts of reovirus (88;259), vesicular stomatitis virus (1), and vaccinia virus (307;308) by virion associated enzymes. Both cap 1 and cap 2 structures are present in mRNAs extracted from cells infected with vesicular stomatitis

virus (195), reovirus (89), and adenovirus (189;275). In eukaryotic cells, it is noteworthy that the complexity of the cap increases as one moves up the evolutionary scale from yeast (cap 0) through slime mold (75% cap 0, 20% cap 1), brine shrimp larvae and sea urchin embryos (exclusively cap 1), silkworm (cap 2), and mammals (high percentage of cap 1 and cap 2).

The mechanisms of cap synthesis were first deduced in 1970's from studies of purified virions of animal viruses which contain all the enzymes for the formation of capped mRNA. Incubation of disrupted vaccinia virus (308), vesicular stomatitis virus (194), or chymotrypsin-digested reovirus (88) in a defined reaction mixture containing ribonucleotides and the methyl donor S-adenosylmethionine, resulted in the synthesis of capped mRNA (Fig. 1-2). In vaccinia virus and reovirus, capping begins with dephosphorylation of the first transcribed nucleotide by nucleotide triphosphatase, and continues with the transfer of the GMP residue from GTP to the 5' diphosphate end of the nascent mRNA to form a unique 5'-5' phosphodiester linkage. A methyl group is then transferred from S-adenosylmethionine (AdoMet) to the N^7 position of the added guanine residue to create a m^7GpppN structure (cap 0) (172;295). Next, a methyl group from AdoMet is transferred to the 2' position of the ribose of the penultimate nucleoside, to form m^7GpppN^m (cap 1). Uncapped polyribonucleotides or even capped ones lacking the 7-methyl group are not methyl acceptors for the 2'-O-methyltransferase, indicating that 2'-O-methylation is the final step in vaccinia mRNA capping (18;19). In vesicular stomatitis virus, cap formation proceeds through transfer of a GDP moiety from GTP to

the 5'-monophosphate end of the acceptor RNA (194) and the ribose-2'-O-methylation seems to precede the guanine-7-methylation (290).

Enzymes that synthesize 5' cap 0 structures have been solubilized and purified from vaccinia (171;172;269) and also from a number of cellular sources (180). The vaccinia guanylyltransferase and N^7-methyltransferase exist as a complex of two polypeptides of molecular weight 95 kD and 33 kD encoded by the viral D1L and D12L genes respectively (184;207). The RNA triphosphatase and guanylyltransferase domains colocalize within the N-terminal of the large subunit (264;267). The methyltransferase domain resides in the small subunit (61;267) and in the C-terminal of the large subunit (118;119;166). Enzyme activities have also been identified from eukaryotic sources, for example, rat liver, HeLa cells, and yeast (180). A distinctive feature of the cellular enzyme is the lack of tight physical association between the guanylyltransferase and 7-methyltransferase activities. These two activities are readily separated during chromatography of cell extracts (180;181). However, the guanylyltransferase and triphosphatase activities are contained within a single polypeptide, which resembles that of the vaccinia capping enzyme. Cellular genes encoding the capping enzymes have been cloned in the past few years (165;180;262;283). Yue et al have identified cDNAs encoding the human and mouse guanylyltransferases, which are the first example of cloned capping enzymes from mammals (322). The mouse and human capping enzymes are 597-aa polypeptides with 95% amino acid sequence identity. The mammalian capping enzymes are bifunctional and consist of an N-terminal RNA triphosphatase domain linked to a C-terminal guanylyltransferase domain. The cellular N^7-

methyltransferase has not been purified to homogeneity and there has been no identification of a cellular gene encoding for the enzyme. The yeast protein is exceptional in that it is a heterodimeric enzyme of two separate polypeptides, 80 kD and 50 kD subunits, which contain guanylyltransferase and triphosphatase activities respectively (126). The gene for the guanylyltransferase has been identified (262).

Cellular enzymes that catalyze the methylation of the 2'-OH of the penultimate nucleoside of capped RNA to form the cap 1 structure, RNA (nucleoside-2'-O-)-methyltransferases, have been detected (13;93;103;104;152;312), but not purified or characterized. However, an mRNA nucleoside-2'-O-methylation activity has been isolated and purified from vaccinia virus (18). Gene J3R has been identified to encode this 39 kD protein (253), which will be addressed in detail in Chapter 5.

The mRNA cap structure plays important roles in: mRNA export from the nucleus (113;289); efficient translation initiation; the protection of mRNAs against nuclease attack (87;263); the initiation of transcription of viral and cellular mRNAs (86;132) and facilitation of mRNA splicing (101;146). Each of these functions is detailed below. A relationship between methylation at the 5' end and the polyadenylation at the 3' end of mRNA, and the role of the 5' capping and 3' polyadenylation in efficient initiation of translation will be addressed later.

The cap structures at the 5' end of mRNA have an important effect on stabilizing mRNAs. Reovirus mRNAs containing m^7GpppN^m or GpppG at the 5' end are degraded more slowly than molecules bearing $pppN^m$, pppN or ppN ends when they are injected into Xenopus oocytes or added to wheat germ or L cell protein synthesizing extracts (87).

Degradation kinetics of molecules containing m^7GpppN^m or GpppG termini are identical. This indicates that blocking of the 5' end with guanosine, without methylation, is sufficient for protection of the mRNA against nucleases. Similar preferential degradation of uncapped RNAs by a wheat germ extract was also established for CPV mRNA (263). However, the presence of a terminal m^7G is not the only factor determining mRNA stability. Viral RNAs which do not contain the caps are active messengers both *in vitro* and *in vivo*. Some of the uncapped RNA molecules may be protected against exonuclease degradation by interaction with proteins or by a specific conformation at their 5' termini.

The presence of a cap increases both the efficiency and the accuracy of pre-mRNA splicing. The cap structure is required for splicing *in vitro* at the level of spliceosome assembly. It also selectively enhances *in vitro* excision of the 5' proximal intron from pre-mRNAs containing multiple introns (218). Capped pre-mRNAs are spliced more efficiently than uncapped precursors when injected into Xenopus oocyte nuclei (124). Although the cap structures enhance splicing efficiency, splicing of uncapped RNAs occurs readily *in vitro* (146).

Capping is also coupled to transcription. It has been suggested that capping seems to occur when mRNA chains grow to between 25 and 50-nt after transcription initiation (132). Similarly, vaccinia capping enzyme cannot access the 5' triphosphate terminus of the growing transcript until it is greater than 27-nt, but the enzyme can readily modify the 5' terminus upon further extension of the transcripts (112). Vaccinia capping enzyme also participates directly in the transcription of viral mRNAs, as a

transcription termination factor in early mRNA synthesis (266) and as an initiation factor during transcription of vaccinia intermediate genes (298).

3' Polyadenylation

Poly (A) was found to be present in mammalian cells in 1960 (80). The significance of poly (A) was not recognized until 1970 when Kates et al reported that poly (A) was covalently linked to the 3' end of vaccinia virus messenger RNA (133;134). Shortly after that, globin mRNA was found to be polyadenylated (158). Since then, many species of mRNA have been examined for the presence of 3' terminal poly (A) and the majority have been found to possess it. Most viral mRNAs are also polyadenylated. Notable exceptions are histone mRNAs (3), and reovirus mRNAs (276). Even among the mRNAs that are generally polyadenylated, there are probably some molecules lacking poly (A). In vaccinia virus mRNA formed by vaccinia virus core *in vitro*, about 5% of the molecules are not polyadenylated (204).

Synthesis of the mammalian poly (A) tails involves a two-step reaction: endonucleolytic cleavage of the primary transcript and polyadenylation of the upstream cleavage product (Fig. 1-3) (182;183). These two reactions are tightly coupled, depend on a signal sequence and require multicomponent machinery including a 4-subunit cleavage/polyadenylation specificity factor (CPSF), a 3-subunit cleavage stimulation factor (CstF), two multisubunit cleavage factors (CF I and CF II), a poly (A) polymerase (PAP), and a poly(A) binding protein (PAB) (54;226). *In vitro* assays of these two reactions show that the hexanucleotide AAUAAA sequence present 10-30 nt upstream of

the cleavage site and a cleavage/polyadenylation specificity factor (CPSF) are required for both phases. The reaction starts as a CPSF specifically binds RNA containing the AAUAAA sequence (130). This binding is greatly enhanced by an interaction with a cleavage stimulation factor (CstF) which binds a much less conserved GU-rich or U-rich sequence 20-40 nt downstream of the cleavage site (163). The two factors that are bound to the nascent RNA form a stable ternary complex, which then recruit the other components to the cleavage site. Then the endonucleolytic cleavage step is carried out by the cleavage factors (CF I and CF II) and poly (A) polymerase as well (282). After the cleavage reaction, the downstream cleavage fragment is rapidly degraded *in vivo* and *in vitro*, and the CstF and cleavage factors are presumed to leave the complex. CPSF and PAP remain bound to the upstream cleavage product and carry out the addition of a short poly (A) tail of about 10-nt. PAP is activated by interacting with CPSF and it binds specifically to mRNA substrates. Lastly, a 50 kD poly (A) binding protein (PAB II) binds to the complex and causes a rapid burst of processive synthesis of a poly (A) tail of about 250-nt. The RNA-protein interaction analysis shows that PAB II, CPSF and PAP form a quaternary complex with the substrate RNA that transiently stabilizes the binding of PAP to the RNA 3' end. Only the complex formed from all three proteins is competent for the processive synthesis of a full-length poly (A) tail (26). It is hypothesized that PAB II works by cooperating with CPSF to hold PAP in place on the primer RNA, and that these interactions are interrupted after the tail has reached a distinct length (301). Poly (A) polymerase, initially purified as a 60 kD protein from calf thymus (303), was later shown by cDNA cloning to have a molecular weight of 82 kD. It is a

single polypeptide and contains putative domains for RNA binding, catalysis and intracellular localization (232). Initial sequence analysis predicted that its amino-terminal two-thirds contains the catalytic activity (232). Recent studies have suggested that two highly structured regions separated by a loop region that are similar to the nucleotidyl transferase family may contain the catalytic function (170).

Synthesis of the vaccinia poly (A) tails is different from the process observed in the mammalian system. Vaccinia poly (A) polymerase activity has been isolated from virus cores (190;192) and from the cytoplasm of the vaccinia-infected cells (37;205;206). The enzyme, with a molecular weight of 80 kD, was purified by DNA-cellulose affinity chromatography over 300-fold from vaccinia cores (191). This 80 kD protein contains two polypeptides with molecular weights of 51 kD and 35 kD when resolved on SDS-acrylamide gel electrophoresis. The genes encoding these two polypeptides (55 kD and 39 kD) were identified to be viral genes E1L and J3R respectively (94). Immunoblots of purified poly (A) polymerase revealed that antibody specific for either *E.Coli* expressed proteins comprising the C-terminal half of the E1L gene product and the complete J3R gene product or synthetic peptides encompassing amino acids 2-15 of the E1L gene product and 2-17 of the J3R gene product reacted specifically with polypeptides of the expected size. The VP55-VP39 heterodimer was relatively stable to column chromatography and glycerol gradient sedimentation, but was dissociated by antibody to an N-terminal peptide of VP55. Poly (A) polymerase activity was associated with immunopurified VP55, but not with immunopurified VP39. However, VP39 binds specifically to free poly (A) and is required for forming long poly (A) and it has a

stimulatory effect on polyadenylation (97). There is no obvious sequence homology between vaccinia virus VP55 and PAP from mammalian cells or yeast, or homology between vaccinia VP39 and PAB II from mammalian cells or yeast. Unlike other eukaryotic systems, no specific sequences such as AAUAAA in the RNA primer is required for viral PAP or other factors to function, though it was documented that both polyadenylation and stable VP55 binding required the presence of multiple uridylates within a 31-40 nt RNA segment (98). Furthermore, there is no precleavage of the pre-mRNA substrate before polyadenylation, and the polyadenylated 3' ends of early mRNA are formed by transcriptional termination rather than a specific RNA cleavage event (246). No peptides of vaccinia other than VP55 and VP39 have been found to play a role in polyadenylation, and no CPSF, CstF or CFs homologues have been identified in vaccinia system. Kinetic studies have revealed that polyadenylation occurs in a biphasic manner (268). *In vitro* polyadenylation assays show that VP55 catalyzes the addition of 30-35 adenylates to an RNA primer in a rapid highly processive burst and then VP39 dramatically stimulates the rate of elongation of the RNA primer possessing poly (A) tails beyond 30-nt (97). The transition from processive to a nonprocessive polyadenylation by VP55 is regulated by the net length of the 3'-oligo (A) rather than by the number of adenylate additions catalyzed by VP55 (96).

mRNA polyadenylation is a universal and functionally significant process. The poly (A) tail influences virtually all aspects of mRNA metabolism, including mRNA stability, translation efficiency, 5' methylation, mRNA splicing, and mRNA transcription. The effect of polyadenylation on translation efficiency and the interaction with the 5'

methylation will be addressed later. Evidence for a role of the poly (A) tail in the regulation of mRNA degradation comes from the observation that deadenylation is the first and rate-limiting step in the breakdown of some unstable mRNAs in mammals and in yeast (196;270). It has also been reported that in eukaryotes the absence of a poly (A) tail is associated with mRNA instability (231).

There is mounting evidence suggesting that splicing and polyadenylation can be linked. A functional polyadenylation signal can enhance splicing of the 5' terminal intron *in vitro*, and vice versa. This result implies that excision of an mRNA's last intron and polyadenylation can be functionally linked (208;306). Recent studies suggest that splicing factors can play auxiliary roles in poly (A) site selection (31;108). Furthermore, in a nuclear extract *in vitro*, a 3' splice site is sufficient to stimulate 3' processing (300). The precise mechanism of the coupling between splicing and 3' processing is not known.

Polyadenylation and transcription termination are coupled. As described in the transcription termination section, coupling of 3' processing and transcription termination is thought to prevent premature termination and thus help to ensure the synthesis of full-length pre-mRNAs. The mechanism of this coupling is currently not known. Recently, Proudfoot and coworkers demonstrated that mutations of cleavage factors rather than polyadenylation factors disrupted transcription termination, which provides fresh insight into this area (27).

Lastly, RNA polymerase is associated with both the polyadenylation machinery and the splicing machinery. McCracken et al found that the cleavage/polyadenylation specificity factor (CPSF) and cleavage stimulation factor (CstF) specifically bound to

affinity columns with the carboxy-terminal domain (CTD) of the RNA polymerase II and copurified with RNA pol II in a high-molecular order complex (176). Combined with the reports about the association between the CTD and the splicing factors (185;324), transcription, splicing and polyadenylation are thought to be coupled.

5', 3'- End Interactions and Translation

The role of caps in facilitating translation was first demonstrated with reovirus and VSV mRNAs, which required 5' terminal m^7G in caps for efficient translation in a wheat germ cell-free protein-synthesis system (34). A similar selection by ribosomes of capped over uncapped mRNAs has been demonstrated *in vitro* by incubating a mixture of $pppG^m$ and m^7GpppN^m ended reovirus RNA molecules in wheat germ extracts (200;201). Removal of 5' terminal m^7G from reovirus mRNA, rabbit reticulocyte mRNA, and silk fibroin mRNA, or bovine parathyroid mRNA resulted in a marked decrease in ability to direct protein synthesis in wheat germ extract. Moreover, the 24 kD cap-binding protein differentially stimulates the translation of capped mRNAs *in vitro*. The cap-binding protein also forms stable complexes with eIF-4A, which is required for attachment of 40S ribosomal subunit to mRNA (281). *In vivo*, in VSV-infected BHK cells, all polysome-associated viral RNA was capped (248). Based on the *in vitro* and *in vivo* findings, it is proposed that the cap structure recruits the small ribosomal subunit (40S subunit) to the mRNA during translation initiation. These activities of the cap structure are dependent upon the eIF4F complex, which binds to the cap structure through the cap binding protein eIF-4E. EIF4F in eukaryotic cells consists of two core subunits, the mRNA cap binding

protein eIF4E and the large subunit eIF4G which contacts eIF3, the 40S subunit-associated factor. In addition, eIF-4A can bind to eIF4G and it is also required for cap-stimulated 40S subunit recruitment (149;225). However, there is no absolute cap requirement for translation because uncapped mRNAs can be translated in cell-free extracts (217). The mRNAs of polio virus and several other eukaryotic viruses lack 5' caps (17), but RNAs from purified poliovirus are translated efficiently in mammalian cell extracts and RNAs isolated from polyribosomes of polio virus-infected HeLa cells did not contain 5' caps (210). It is proposed that in picornaviruses, IRES elements can provide an alternative means to stimulate 40S subunit binding to mRNA (128).

While a lot of data has accumulated on the possible functions of 5' terminal 7-methylguanosine, little is known about the role of the ribose methylations present in the second and third nucleotides from the 5' end of many mRNAs. Studies of Muthukrishnan et al (200-202) indicate that 2'-O-methylation of the penultimate nucleoside may have a positive influence on mRNA binding to ribosomes. Most of the evidence in favor of this possibility comes from the binding experiments with synthetic polynucleotides bearing m^7GpppG^m or m^7GpppG termini (201;202). 2'-O-methylation has been shown to have a stimulatory effect on polymer binding to ribosomes using these synthetic polynucleotides. In contrast, little or no preference for 2'-O-methylated mRNAs to bind to wheat germ or rabbit reticulocyte ribosomes has been observed when capped vaccinia virus mRNAs bearing the m^7GpppN^m or m^7GpppN termini are employed in the assay (202). This discrepancy can be explained as different ribosome binding affinities of synthetic nucleotides compared to authentic vaccinia mRNAs. However, a

recent report from Richter and coworkers comparing injection of mRNAs containing cap 1 and cap 0 structures indicates that cap ribose methylation of c-mos mRNA is important for translational recruitment and for the progression of the oocyte through meiosis (148). It is not known whether ribose methylation will affect the interaction between the cap structure and the eIF-4F complex or whether it will facilitate the binding of the 40S ribosomal subunit.

A series of independent experiments performed during the late 1980s and early 1990s led to the conclusion that the poly (A) tail was capable of stimulating the translation of mRNA (129). First, in reticulocyte extracts, poly $(A)^+$ mRNA has a translational advantage over poly $(A)^-$ mRNA with respect to translation initiation efficiency (84). Translational preference for poly $(A)^+$ mRNAs was also observed in other cell-free extracts, including yeast (250;251). Second, it was shown that the addition of the poly (A) tail to mRNA stimulated its translation in the amphibian oocyte (91;222;260;294). Messenger RNA translation was inhibited when the addition of the poly (A) tail was blocked either by mutating the polyadenylation signal on the mRNA or by chemically modifying the 3' ends of the mRNA. Third, a large set of mRNA electroporation experiments revealed that the poly (A) tail acted as an enhancer of mRNA translation (92). Finally, genetic experiments in the yeast *Saccharomyces cerevisiae* revealed that the poly (A) tail binding protein, Pab1p, was required for efficient mRNA translation and both 40S binding and 60S joining was implicated as the target of poly (A) tails in translation (286). It was shown that a 2 fold increase in mRNA translation by the poly (A) tail in rabbit reticulocyte lysates was due to a stimulation of the joining of the

60S ribosomal subunit to the mRNA (197;198). Consistent with this result, extragenic suppressors of Pab1p *ts* mutation showed alterations of the 60S ribosomal subunit (250). It was also found that the stimulation of translation by the poly (A) tail in the yeast extracts required Pab1p using monoclonal antibodies to Pab1p (286). It was shown in this report that the poly (A) tail stimulated mRNA translation by enhancing the binding of the 40S small ribosomal subunit to the mRNA. Furthermore, it was found that Pab1p was associated with eIF4F through eIF4G, and that this contact required the presence of RNA (287). Recently, it was shown that the physical interaction between Pab1p and eIF4G resulted in the recruitment of the 40S subunit to mRNA *in vitro* (288). Based on the experiments above, it was proposed that the poly (A) tail on mRNA stimulates the recruitment of the 40S subunit during translation via the interaction of Pab1p with eIF4G. How poly (A) tail stimulates the recruitment of 60S subunit is not unknown yet.

Since both the cap and the poly (A) tail bind to the common target eIF4G, a closed-loop model was proposed (Fig. 1-4) (129). In this model, mRNA is in a transient pseudo-circular structure mediated by the interaction of Pab1p and eIF4E with eIF4G. However, a direct demonstration that the interaction of factors associated with 5' and 3' ends of an mRNA can allow for mRNA circularization has not yet been achieved. In addition, how poly (A) tails stimulate translation initiation in the absence of the cap structure is unknown. Furthermore, the similar functional interaction between Pab1p and eIF4G from higher eukaryotes has not yet been reported.

Vaccinia Virus, A Good System to Study Transcriptional Regulation

Vaccinia virus, the prototypic orthopoxvirus, is a double-stranded DNA virus which replicates its 192kb genome in the cytoplasm of the host cell. This replication strategy requires a host independent transcription system including DNA-dependent RNA polymerase, associated transcription factors, a primer-dependent poly (A) polymerase and capping enzymes. Vaccinia mRNAs have typical eukaryotic features including a poly (A) tail and a methylated cap. Thus, vaccinia virus presents itself as an attractive model system for studies of eukaryotic transcriptional regulation.

Vaccinia Virus Life Cycle

Vaccinia virus infection starts with fusion of the virus lipid bilayer with the host cell membrane, followed by release of the virus core containing the viral genome into the cytoplasm of the infected cells. Following the initial uncoating, viral gene expression occurs in a cascade: early genes are expressed prior to viral DNA replication, and intermediate and late genes are transcribed only after DNA replication has begun. The components for early transcription are packaged in the virion so that mRNA synthesis initiates immediately after entry into the cytoplasm (135;199). The subsequent synthesis of DNA polymerase and other viral early proteins leads to DNA replication. Some of the early proteins encode intermediate transcription factors which are needed for transactivating intermediate genes. Intermediate genes in the replicating DNA are transcribed and the mRNAs are translated into intermediate proteins, some of which are

late transcription factors. Late proteins include structural proteins, and early transcription factors which are all packaged in the virion for subsequent infection (Fig. 1-5).

Viral mRNAs produced in infected cells or by virus cores are capped (32;308), and polyadenylated (133;134), with no indication of splicing. The early mRNAs are of discrete size due to the specific transcription initiation and termination, while the intermediate and late mRNAs are heterogeneous in length due to non-specific transcription termination.

All three classes of vaccinia genes are transcribed by the same virus-coded multisubunit RNA polymerase. Similar to eukaryotic RNA polymerase II, the vaccinia RNA polymerase has a molecular mass of 500 kD with two large (RPO147 and RPO132) and many small subunits (20). The largest two subunits are homologous to the corresponding subunits of cellular RNA polymerase, and are 30% identical to the largest subunits of the *Saccharomyces cerevisae* RNA pol II, RPB1 and RPB2. The smallest subunit RPO7 is 23% identical to the smallest subunit of yeast RNA polymerase II. Another subunit RPO30 has 23% sequence identity to eukaryotic transcription elongation factor SII over a 180-amino acid region. No sequence resemblance to eukaryotic or prokaryotic RNA polymerase has been found in the remaining subunits of vaccinia RNA polymerase. The genes encoding the two large subunits and six of the small subunits (RPO35, RPO30, RPO22, RPO19, RPO18 and RPO7) have been identified (5;9;40;223). Vaccinia RNA polymerase by itself can transcribe single-stranded DNA templates, but additional transactivating factors are required for specific initiation of transcription of double-stranded DNA templates at early, intermediate and late promoters.

Vaccinia gene expression is controlled primarily at the stage of transcription initiation using specific trans-acting factors required for each class of viral promoters. The cis- and trans-acting factors for vaccinia virus transcription have been defined by biochemical and genetic analysis. The promoter sequences for early, intermediate and late genes have been studied by deletion mutagenesis (186). The promoter elements of three classes are about 30 nt in length extending about 30 nt upstream of the RNA start site. Three domains are defined: the initiator, the spacer and the core region (Fig. 1-6). The early promoters contain a degenerate initiator sequence A/G, a spacer region of about 10-nt (-5 to -10), and a core region of 15-nt (-15 to -30). The nucleotides in the promoter element which do not tolerate substitutions are indicated in Fig 1-6. (71;72). Only five intermediate promoters have been identified and compared. The intermediate promoters contain a more stringent initiator sequence TAAA positioned at the RNA start site, a 10-11 nt spacer region (-4 to -14), and a 14 nt core element (-15 to -28). The nucleotides that have a strong positive effect on transcription are indicated in Fig.1-6 (15). The late promoters contain the most stringent initiator sequence TAAATG/A and the importance of each nucleotide has been verified by mutagenesis (71;72). The core region of the late promoters, containing the A/T rich sequence, is less stringent. No enhancer elements have been identified in vaccinia virus. The trans-acting factors for each class will be described in each section of viral gene expression below.

Vaccinia Virus Early Gene Expression

Early gene expression is initiated from the infectious virion immediately following infection before the virus genome is released into the cytoplasm. Early mRNA is detected 20 min post infection and peaks within 1-2 hrs. The transcription machinery required for early gene expression is contained in the vaccinia virion. Soluble virion extracts can transcribe exogenous DNA templates containing early promoter sequence added *in vitro* (100;245). The soluble virion extracts contain two critical components, RNA polymerase and the early transcription factor VETF (43). Purified RNA polymerase lacks the ability to transcribe double stranded DNA templates unless complemented with VETF (43). RNA polymerase isolated from vaccinia virions comprises two forms, a core polymerase and a holoenzyme which contains the core enzyme in association with an 85 kD polypeptide encoded by vaccinia gene H4L (RAP94) (4;7). Only the holoenzyme can initiate transcription from a double-stranded DNA template containing an early promoter in conjunction with VETF. However, the core polymerase lacking RAP94 can transcribe a single-stranded DNA template or double stranded DNA templates containing intermediate and late promoters with specific transcription factors (313). Thus, the H4L gene product behaves like an early gene transcription factor which is integral or tightly associated with the RNA polymerase. VETF is a heterodimer of 82 and 70 kD subunits encoded by vaccinia genes A8L and D6R respectively (38;95). A8L has zinc finger and leucine zipper motifs whereas D6R contains a motif associated with ATP binding and ATPase activity. VETF binds specifically to the core region of early promoter (-12 to -29) and DNA downstream of

RNA start site (+8 to +10) as deduced by DNA footprinting (39). Transcription is reduced *in vivo* and *in vitro* with single-base substitutions in the promoter core region (42;323). It has also been shown that VETF has a DNA-dependent ATPase activity which is associated with the subunit encoded by D6R. ATP accelerates the dissociation of VETF from its binding site on the promoter (39). This activity is not required for promoter binding but is essential for transcription, possibly via a promoter clearance mechanism. The formation of transcription initiation complexes on early promoters was addressed (14;39;42;43). The order of the assembly was demonstrated by incubating vaccinia virus VETF and RAP94 associated RNA polymerase with an early promoter template immobilized on paramagnetic beads in stepwise addition experiments (14). The binding of VETF to early promoters is likely to be the first step in assembly of the complexes, followed by RNA polymerase. Therefore, initiation of early vaccinia transcription requires, in addition to the RNA polymerase, the early transcription factors VETF, and the RNA polymerase associated protein, RAP94.

The 3' ends of early mRNAs are formed by transcription termination, which is dictated by a cis-acting sequence UUUUUNU in the nascent RNA chain (268;323). Mutagenesis studies indicate that 3' end formation generally occurs 20-50 nt downstream of this cis-acting sequence (323). Purified vaccinia RNA polymerase and VETF cannot terminate in response to the UUUUUNU signal *in vitro*. Therefore, a separate vaccinia enzyme is required for termination. To determine the trans-acting factors for termination, column fractions from soluble vaccinia virions were added to purified RNA polymerase and VETF in a complementation assay. Using this assay, the complex responsible for

early gene termination was defined as the heterodimeric viral capping enzyme encoded by gene D1L, D12L (266) and at least one other protein, factor X (73;266), subsequently defined as D11L. The mechanism of action of the termination factors (VTF) has been studied extensively. The capping enzyme elicits termination through interactions with the nascent RNA (110). Contacts between the capping enzyme and the nascent RNA have been localized by UV crosslinking to the large capping enzyme subunit D1 (110). The 5' cap structure is not required in any way for transcription termination (110). The D1 subunit by itself, which is fully active in triphosphatase and guanylyltransferase functions, has no demonstrable VTF activity *in vitro*. The methyltransferase domain of D1 and D12 subunits also has no VTF activity. Therefore, both full-length subunits are required for transcription termination. Mutations that abolish enzyme-GMP complex formation or methyltransferase activity have no effect on the termination activity (162). Thus, there must exist a domain for termination distinct from the catalytic domains for nucleotidyl transfer and methyl transfer. One the other hand, the capping enzyme can also form a complex with vaccinia RNA polymerase in solution in the absence of nucleic acid (111). The RNA polymerase-capping enzyme complex does not have to assemble at a promoter since paused transcripts will elongate and terminate at the appropriate sequence when exogenous capping enzyme is added at a pause site (161). VTF-mediated termination requires energy and termination is coupled to the hydrolysis of ATP (110;266). It has been shown that the ATP-hydrolysis activity of termination is not associated with capping enzyme (321), but rather with Factor X (75). Factor X corresponds to vaccinia nucleoside triphosphate phosphohydrolase-I (NPH-I), the product

of gene D11L and a DNA-dependent ATPase of DExH-box family. NPH-1 is tightly associated with the transcription termination complex and functions to catalyze release of UUUUUNU-containing nascent RNA from the elongation complex in concert with capping enzyme (75). However, NPH-1 does not contact nascent transcripts by a UV cross-linking study. The function of NPH-1 in early transcription termination is further confirmed by using ts mutants in gene D11L. Transcription-competent cell-free extracts of D11L *ts* mutant lack signal-dependent early gene transcription termination activity, which can be restored by the addition of either free NPH-1 or a GST-NPH I fusion protein (50). In summary, the termination site choice and the efficiency of termination are determined by a kinetic balance between the rate of signaling and the rate of polymerase movement. Signaling rate is dictated by the concentration of hydrolyzable ATP (110). Elongation rate is influenced by the concentration of NTPs and also by the intrinsic pausing signals in DNA template sequences (74). Although the molecular details of termination are not known, the model suggests that the capping enzyme scans the U_5NU sequence of nascent RNA and then activates ATP hydrolysis by NPH-1, resulting in release of the nascent RNA from the RNA polymerase.

Termination of vaccinia early transcripts bears some resemblance to rho-dependent termination in *E.Coli* (241). First, a cis-acting signal occurs within the RNA and termination occurs downstream in both systems. However, the C-rich signal for rho is less well defined than the U_5NU signal used by vaccinia virus. Second, NTP hydrolysis is required in both systems. However, rho factor carries RNA signal recognition and NTP hydrolysis functions in *E.Coli*, whereas capping enzyme recognizes

termination signal in RNA and NPH-I hydrolyzes NTP. In addition, rho factor displays an RNA-dependent ATPase and helicase activity, whereas NPH-I is a DNA-dependent ATPase with no apparent helicase activity.

Vaccinia Virus Intermediate Gene Expression

Intermediate gene expression occurs after initiation of DNA replication and before expression of the more abundant late genes. Intermediate mRNA is detected at 100 min following a synchronous infection in HeLa cells. Trans-acting factors for intermediate gene expression are early gene products and are made by the virus prior to viral DNA replication. Therefore, an *in vitro* system using cytoplasmic extracts from cells infected with vaccinia virus in the presence of a DNA replication inhibitor has been developed for studying intermediate transcription (297). In addition to RNA polymerase, at least two factors (VITF-A and VITF-B) have been identified which can reconstitute transcription of templates containing intermediate promoters in this *in vitro* transcription system. VITF-A was identified as virus capping enzyme (298). The role of capping enzyme in transcription initiation maybe related to its ability to bind RNA polymerase (41;111;297). Intermediate gene transcription utilization is independent of RNA guanylylation (114). VITF-B can form a template-committed complex in the absence of RNA polymerase and VITF-A, and the complex is resistant to challenge with a second promoter template (297). VITF-B also displays ATPase activity and is required for promoter melting (297). VITF-B can be separated into two components, and at least one component is virus-coded (297). Since the initial characterization from the Stunnenberg

lab, Moss and coworkers have reported the purification of two factors (VITF-1 and VITF-2) that are required for intermediate gene transcription in addition to RNA polymerase and capping enzyme (247). VITF-1 is encoded by the viral gene E4L, which encodes a 30-kD subunit of RNA polymerase (RPO30). RPO30 is a subunit of vaccinia RNA polymerase and has homology to the eukaryotic transcription factor TFIIS. VITF-2 is a cellular protein. The activity of VITF-2 is found in the nuclei of uninfected HeLa cells and in the cytoplasm of other permissive host cells (247). Both groups show that capping enzyme can transactivate intermediate gene expression, but the relationship among other factors found by these two groups is not known. Recently, an intermediate transcription factor (VITF-3) has been identified which is a heterodimer of 100 kD encoded by A23R and A8R (Moss et al, unpublished). Experiments showed that transcription activity is increased *in vitro* when A8R and A23R proteins are synthesized together using pET bacterial expression system. In conclusion, trans-activation of intermediate genes requires capping enzyme, E4L gene product, one cellular factor and the new A23R-A8R heterodimer.

The 3' end formation of intermediate gene transcripts is strikingly different from that of early genes. RNA polymerase, capping enzyme and VITF-B fail to recognize the T_5NT signal and instead produce runoff transcripts *in vitro*, implying differences in the mechanisms used (297). Intermediate mRNAs isolated from infected cells are of heterogeneous length and their 3' ends do not map to the early gene termination sequence (16). This termination pattern bears some resemblance to that of eukaryotic transcription termination.

Vaccinia Virus Late Gene Expression

Initiation of transcription of late genes follows initiation of transcription of intermediate genes. Late mRNA is detected at about 140 min after infection and continues for about 48 hrs in a HeLa cell infection. A capped 5' poly (A) tract of about 35 nucleotides is a characteristic feature of late and intermediate mRNAs (6;256). The poly (A) leader is not encoded by the genome and probably arises by RNA polymerase slippage when initiating within the highly conserved initiator sequence TAAAT. The 5' poly (A) leader was diminished in length when the T residues of TAAAT were mutated, and the leader was absent or limited to a few nucleotides when any of the three A residues were mutated (72). The role of the capped 5' poly (A) leader is not known yet. It is speculated that it might be involved in binding initiation factors and 40S ribosomal subunit, which would then scan to the first AUG.

A reversed genetic approach has been used to identify late transcription factors (136). Cells were infected with vaccinia virus in the presence of a DNA replication inhibitor, which allows only early gene expression. Cells were then co-transfected with a plasmid containing a reporter gene controlled by a late promoter and with various vaccinia virus DNA fragments. Expression of the reporter gene is dependent on infection with vaccinia virus. The transcription of this reporter gene can be blocked by an inhibitor of DNA replication because late gene expression is coupled to DNA replication. Therefore, the reporter gene will be expressed only if the missing trans-acting factors are synthesized from the transfected vaccinia virus DNA fragments. In this way, three intermediate genes A1L, G8R and A2L encoding 17, 30, and 26 kD proteins were

identified as being necessary and sufficient for late gene transcription (123;137;145;315). Though A1L and A2L contain zinc finger motifs, no evidence has been shown that these two proteins mediate late promoter binding activity. One other factor, originally isolated as factor P3, has been identified as the product of the early-late gene H5R and is named VLTF-4 (144;145). VLTF-4 is synthesized before and after viral DNA replication, which is different from other VLTFs. Recently, two independent groups discovered a cellular protein which binds late promoters and activates transcription *in vitro* (314;327). The cellular factor found by Wright and coworkers was both in uninfected HeLa cell cytoplasmic extracts and infected cells, and it copurified with a late promoter-specific DNA-binding activity. The factor found by Broyles and coworkers was isolated from uninfected HeLa cells. It exhibits high specificity for late promoters and transactivates late gene transcription. Both proteins have affinity for Ni-agarose, behave similarly upon chromatography on phosphocellulose, bind late promoter and activate transcription *in vitro*. Whether these two proteins are the same remains to be determined. In conclusion, expression of the intermediate genes A1L, A2L, G8R, an early-late gene H5R (144), and one cellular factor supplies the factors for trans-activating late gene expression.

While factors for initiation of late gene transcription have been well defined, little is known concerning transcription termination. Most late transcripts are long and heterogeneous, lacking defined 3' ends (65;66). Therefore, transcription termination of late genes should be very different from termination of early gene transcription, and may be similar to that of intermediate genes. Experiments from our laboratory suggest that the A18R and G2R gene products influence elongation and/or termination of late and

intermediate transcripts (28;60). The A18R gene, which we believe is involved in transcription termination of postreplicative genes is characterized in this study.

Vaccinia Virus Genetics

Genetic analysis of vaccinia virus provides a way to study the control of gene expression, mRNA processing, DNA replication and DNA recombination. Conditional lethal mutants in different complementation groups have been isolated, including ts mutants and drug dependent mutants. These mutants have been screened for the ability to synthesize viral DNA and viral proteins (58;59). The phenotypes were grouped as normal, DNA-, defective late, or abortive late. No defective early phenotype has been found. The normal phenotype displays viral DNA and protein synthesis which is indistinguishable from wild type virus. The DNA- phenotype refers to mutants which are incapable of [^{3}H] incorporation into DNA under nonpermissive conditions. The defective late phenotype refers to mutants which have abnormal late protein synthesis, whereas the abortive late phenotype refers to mutants which have premature cessation of late protein synthesis under nonpermissive conditions. None of these viruses produce progeny when grown under nonpermissive conditions.

Three of the complementation groups represented by the genes A18R, G2R and J3R are currently under investigated in our lab. The anti-poxviral drug IBT dependent G2R and J3R mutants display a defective late phenotype under nonpermissive conditions. The temperature sensitive A18R mutants show an abortive late phenotype at nonpermissive temperature (220). Further *in vivo* characterization of these mutants

suggests that they are defective in viral gene expression, specifically these proteins are involved in the regulation of intermediate and late gene transcription elongation and termination.

Summary

Little is known about transcription elongation and termination compared to the understanding of transcription initiation, especially in vaccinia virus postreplicative gene expression. Vaccinia early transcripts are of discrete size due to specific transcription initiation and termination. However, intermediate and late transcripts are heterogeneous in length and the 3' end formation is relatively difficult to study. Previous results from our laboratory suggest that the vaccinia A18R and G2R gene products affect intermediate and late transcription *in vivo*. The A18R gene product is a DNA-dependent ATPase and DNA helicase. a temperature-sensitive mutant of A18R displays "promiscuous transcription" at late times post infection. The anti-poxviral drug isatin-β-thiosemicarbazone (IBT) –dependent G2R deletion mutant displays defective late phenotype with respect to RNA synthesis and protein synthesis. The goal of this thesis is twofold. First is to investigate the function of a vaccinia gene A18R product in transcription elongation and/or termination of intermediate and late genes. Second is to identify other genes, like G2R and J3R, which might be involved in the same pathway as the A18R gene product to regulate transcription elongation and 3' end formation. Specifically, we hypothesize that the A18R gene product functions as a negative

transcription elongation factor or termination factor, and G2R and J3R function as positive elongation factors for postreplicative genes.

Fig. 1-1. Model of the assembled phage lambda N antitermination complex. This figure was taken from (69). This figure shows the key protein-protein and protein-RNA interactions among the phage lambda N antitermination complex.

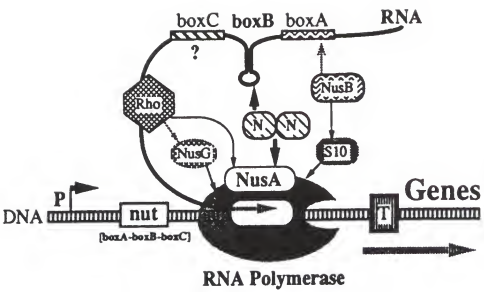
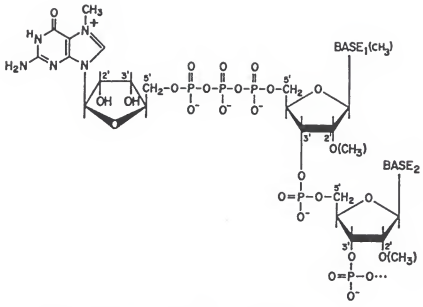

boxC boxB boxA
RNA
?
Rho
NusB
N
N
NusG
S10
NusA
Genes
P
DNA
nut
T
[boxA-boxB-boxC]
RNA Polymerase

Fig. 1-2. Structure of the 5' cap and mechanism of cap formation. This figure was taken from (90). The figure illustrates the 5' end of nascent RNA and cap 0, cap 1($BASE_1$), cap 2 ($BASE_2$)structures. The formation of 5' caps is carried out in four steps via the functions of nucleotide phosphohydrolase, guanylyltransferase, methyltransferase 1 and RNA (nucleoside-2'-O-)-methyltransferases (methyltransferase 2).



$$\text{pppG} + \text{pppC} \xrightarrow{\text{RNA polymerase}} \text{pppGpC} + PP_i \quad (1)$$

$$\text{pppGpC} \xrightarrow{\text{Nucleotide phosphohydrolase}} \text{ppGpC} + P_i \quad (2)$$

$$\text{pppG} + \text{ppGpC} \xrightarrow{\text{Guanylyltransferase}} \text{GpppGpC} + PP_i \quad (3)$$

$$\text{GpppGpC} + \text{Adomet} \xrightarrow{\text{Methyltransferase 1}} m^7\text{GpppGpC} + \text{Adohcy} \quad (4)$$

$$m^7\text{GpppGpC} + \text{Adomet} \xrightarrow{\text{Methyltransferase 2}} m^7\text{GpppG}^m\text{pC} + \text{Adohcy} \quad (5)$$

Fig. 1-3. Diagram of mammalian pre-mRNA 3' end processing. This figure was taken from Keller (139). The figure represents the steps involved in the 3' polyadenylation of mammalian pre-mRNA. Pre-mRNA contains two critical sites for polyadenylation, AAUAAA site, which is 10-35 nt upstream from the poly (A) site, and GU rich site, which is 50 nt downstream from the poly (A) site. 3' end formation comprises of two steps: cleavage and polyadenylation. It starts with cleavage complex formation at the two critical sites in the pre-mRNA including the factors PAP, CPSF, CstF, and CFs. Cleavage occurs and the upstream cleavage product is further polyadenylated by the function of PAP, CPSF and PABII.

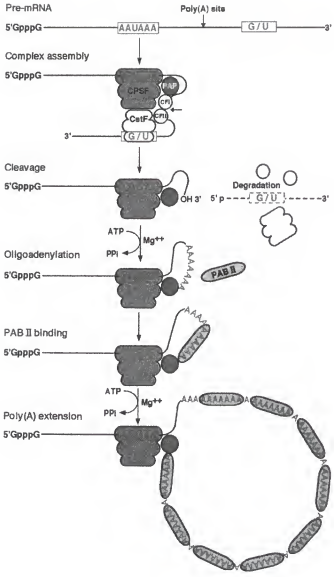

Pre-mRNA
Poly(A) site
5'GpppG
AAUAAA
G/U
3'
Complex assembly
5'GpppG
CPSF
PAP
CPI
CstF
CPI
3'
G/U
Cleavage
5'GpppG
OH 3'
5' p
G/U
3'
Degradation
ATP
Mg++
PPi
Oligoadenylation
5'GpppG
PAB II
PAB II binding
5'GpppG
ATP
Mg++
PPi
Poly(A) extension
5'GpppG

Fig. 1-4. Model for poly (A) stimulated translation initiation. Translation initiation factor eIF4F consists of two subunits: eIF4E and eIF4G. Binding of Pab1p to eIF4G results in the recruitment of the 40S subunit to mRNA. The association of eIF4E with the cap structure could lead to transient or stable mRNA circularization and placement of the 40S subunit near the 5' end of the mRNA.

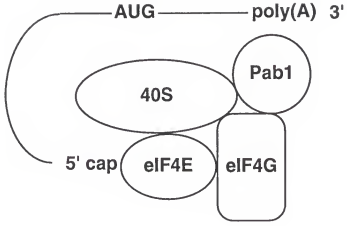

AUG
poly(A) 3'
Pab1
40S
5' cap
eIF4E
eIF4G

Fig. 1-5. Vaccinia virus life cycle. This figure was taken from Fields' Virology (187). It illustrates the infection of virus in host cells from attachment, entry, replication, assembly and budding.

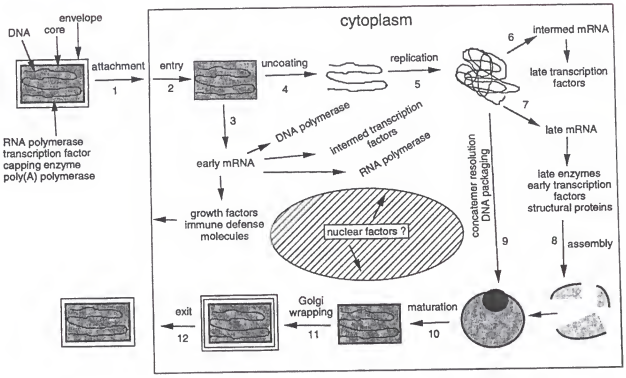

envelope
DNA
core
attachment
1
RNA polymerase
transcription factor
capping enzyme
poly(A) polymerase
cytoplasm
entry
2
uncoating
4
replication
5
6
intermed mRNA
late transcription
factors
7
late mRNA
late enzymes
early transcription
factors
structural proteins
8
assembly
3
DNA polymerase
intermed transcription
factors
RNA polymerase
early mRNA
growth factors
immune defense
molecules
nuclear factors ?
concatemer resolution
DNA packaging
9
maturation
10
Golgi
wrapping
11
exit
12

Fig. 1-6. Early, intermediate, and late promoter sequences. This figure was taken from Fields' Virology (187). The promoter sequence contains the initiator, the core region and sequences between them (the spacer). Nucleotides that have a strong positive effect on transcription are illustrated. The positions of nucleotides are relative to the RNA start site (+1).

VACCINIA VIRUS PROMOTER SEQUENCES

	CORE	INITIATOR
EARLY	AAAA T GAAA TA	A/G
INTER	T TT AAA AA	TAAA
LATE	A/T-rich	TAAATG/A
	-30 -20 -10	+1

CHAPTER 2
IN VIVO ANALYSIS OF A18R FUNCTION IN REGULATION OF VACCINIA VIRUS POSTREPLICATIVE GENE TRANSCRIPTION

Introduction

The vaccinia A18R gene encodes a 56 kD protein that is present in the virion. The A18R gene is expressed both early and late during infection. Previous northern blot analysis showed that the A18R mRNA was detected initially at 1 hr post infection and was observed throughout the late phase of infection (221). Consistent with this mRNA expression profile, an immunoprecipitation analysis of metabolically pulse-labeled vaccinia virus-infected cells also showed that the A18R protein was present initially at 2 hr post infection in small amounts, and this protein synthesis continued throughout the late phase of infection.

A18R protein is a member of the DExH helicase superfamily II. It is a DNA binding protein that has DNA-dependent ATPase and DNA helicase activity. It has limited but provocative sequence homology to ERCC3, a subunit of the mammalian TFIIH. The ATPase activity of A18R is stimulated by both single- and double-stranded DNA but not by RNA (22). The helicase activity of A18R is restricted exclusively to DNA:DNA hybrids of less than 25 bp and proceeds in the 3' to 5' direction (272).

The A18R gene is essential for virus infection. Temperature sensitive mutants in the A18R gene isolated in our lab, C*ts*22 and C*ts*23, can only grow at the permissive temperature of 31^0C, but not at the nonpermissive temperature of 40^0C. The stability of the A18R protein expressed by the A18R *ts* mutant C*ts*23 at both permissive and nonpermissive temperatures is significantly decreased compared to wild type A18R protein (271). The A18R gene plays a role in viral transcription during both early and late phases of infection. The role of A18R in early infection is demonstrated in an *in vitro* virion directed transcription assay. Specifically, it was shown that transcription from NP-40 permeabilized A18R mutant virions is defective at both 31^0C and 40^0C (271). However, A18R has not been isolated from an early transcription complex and no other evidence suggests that A18R transactivates early gene transcription *in vitro*. Late during viral infection, the A18R *ts* mutants exhibit promiscuous transcription, defined as follows (21). Northern blot analysis using D9R and G2R early gene-specific probes was performed to measure the steady state mRNA synthesis pattern in virus infected cells. In a wild type infection, discrete early mRNAs of the expected sizes appeared early and then disappeared at late times. However, in an A18R mutant infection, steady state RNAs were detected by these early probes as heterogeneous bands at late times post infection and some of these RNAs are shorter than normal chain length (21). Thus promiscuous transcription is defined as transcription detected late during infection in regions of the genome which are normally transcriptionally silent.

Promiscuous transcription has profound secondary consequences for a C*ts*23 infection. Because both strands of the DNA genome are utilized in transcription and

because promiscuous transcription can arise from the complementary strands of the genome, significant amounts of double-stranded RNA (dsRNA) are formed late during a C*ts*23 infection. A two-fold increase in the intracellular concentration of dsRNA was detected in C*ts*23 infection compared to a wt infection (21). The dsRNA in turn activates the cellular 2-5A pathway leading to RNase L catalyzed rRNA and mRNA breakdown and results in cessation of protein synthesis at late times post infection (220). The mechanisms and biological effects of the cellular 2-5A pathway will be detailed in Chapter 4. The RNase L mediated RNA degradation presents a challenge for further transcriptional analysis, since the size of native intermediate and late transcripts cannot be measured in an A18 mutant infection.

Three hypotheses could account for promiscuous transcription: (1) reactivation of an early gene promoter late during infection, (2) random, promoter-independent transcription initiation throughout the genome or (3) readthrough transcription from upstream promoters. Northern blot and RNase protection assays conducted in our laboratory by Dr. David Simpson, were used to address the first two hypotheses (317). First, RNase protection experiments indicate that protected fragments for early and intermediate promoters appear with similar kinetics and in similar amounts in the C*ts*23 infection compared to the wt infection. The late F17R promoter turns on at a similar time, but the signal is reduced in intensity and decays prematurely at 40^0C in C*ts*23 infection. Most importantly, the early promoter is not utilized at late times in the A18R mutant infection at 40^0C. Significantly, an RNase protection assay, used to measure the promoter activity of a promiscuously transcribed early gene, M2L, showed that this

promoter is also not utilized at late times in an A18R mutant infection at 40^0C, thereby contradicting the hypothesis that promiscuous transcription represents reactivation of early promoters late during infection. Second, northern blot analysis was performed to determine whether random initiation occurs throughout the virus genome during A18R mutant infections. The kinetics of mRNA synthesis observed are similar to those detected using RNase protection. Importantly, the early C11R mRNA signal disappears at late times, confirming that the early promoter does not reactivate late during infection. In *Cts*23 infected cells at 40^0C at late times, some G8R transcripts are shorter than normal, indicating the expected RNase L catalyzed breakdown of RNAs. Consistent with the RNase protection analysis described above, the late F17R gene seems to be poorly transcribed in A18R mutant infections at 40^0C. Most importantly, promiscuous transcription is not observed with either the early or the late gene probe at late times post infection, thereby contradicting the hypothesis that promiscuous transcription represents completely random initiation within untranscribed regions of the genome.

We designed the present study to test the readthrough hypothesis. The results reported here show that mutation of the A18R gene results in increased readthrough transcription from an upstream intermediate promoter. Furthermore, we show that A18R mutant infection of RNase L knockout mouse fibroblast cells (KO3) does not result in 2-5A pathway activation, yet the virus mutant is still defective in late viral gene expression and remains temperature sensitive. These results support the hypothesis that the A18R gene product is a negative transcription elongation factor and/or a termination factor for intermediate and late viral genes.

Materials and Methods

Cells and Virus

The continuous African green monkey kidney cell line BSC40 and conditions for cell culture have been previously described (58;59). KO3 cells are immortalized fibroblasts established from an RNase L knock out mouse. They were grown in Eagle's Modified Medium (Life Technologies, Gaithersberg, MD) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc, Logan, UT) and antibiotics. A549 cells, a human lung carcinoma cell line, were obtained from Dr. Sue Moyer and maintained in Dulbecco's Modified Eagle's Medium containing 10% Fetal Bovine Serum and antibiotics. Wild-type vaccinia virus strain WR, and gene A18R *ts* mutant virus C*ts*23, the conditions for their growth, infection, and plaque titration have been described previously (58;59)

Plasmids

PCFW10 (316), which contains the vaccinia virus 11K (gene F17R) late promoter placed upstream of the 375-nt G-less cassette in , was obtained from Dr. Cynthia Wright (University of South Carolina). pVGFG (57) contains the vaccinia virus growth factor (VGF, gene C11R) early promoter (39) placed upstream of the 375-nt G-less cassette in pC_2AT19. PG8G (57) contains the vaccinia virus gene G8R intermediate promoter (15) placed upstream of the 375-nt G-less cassette in pC_2AT19. pK2G, which contains the vaccinia virus gene K2L promoter placed upstream of the 375-nt G-less cassette in

pC_2AT19, was constructed as follows. Two complementary K2L promoter-containing oligonucleotides were synthesized, 5' phosphorylated, annealed, and ligated to pC_2AT19 linearized with SacI and blunt ended with T4 DNA polymerase. The inserted oligonucleotide has the sequence

5' *AGTACT*AACATAAAAATAAGGTTAATTATTAATACCATAAAATCAT 3'

where the plain text represents the K2L promoter, the first two nucleotides of the translation initiation ATG are underlined, and the italic text represents a ScaI site introduced for ease of identification of the desired clone. pK4G, containing the vaccinia virus gene K4L promoter, was constructed in the same fashion as pK2G, except that the sequence of the inserted oligonucleotide was

5' *AGTACT*GAGTGAAGTGATATAGGATTATTCTTTTAACAAATAAAAT 3'. The inserts in both pK4G and pK2G were sequenced to confirm their identity.

Riboprobes

Riboprobes were synthesized as described (Promega Protocols and Applications Guide), using as a template for T7 or SP6 RNA polymerase either linearized plasmid DNA (pGEM-VGF, pGEM-30K, pGEM-11K and pGEM-M2L) or PCR products obtained by amplification of desired regions of wild-type vaccinia DNA. Plasmids pGEM-VGF, pGEM-30K and pGEM-11K were kindly provided by Bernard Moss (NIH), plasmid pGEM-M2L was kindly provided by Dr. Richard Moyer (University of Florida). Templates produced by PCR were constructed using primers complementary to indicated vaccinia sequences. The downstream primer contained the consensus T7 RNA

polymerase promoter sequence, 5' tgTAATACGACTCACTATA 3', where the uppercase letters represent the T7 RNA polymerase promoter and the lowercase letters represent extra nucleotides necessary for efficient T7 RNA polymerase binding. PCR reaction products were purified on 10% polyacrylamide 50% urea gels.

Isolation of RNA

RNA was extracted from infected cells and purified by RNeasy Total RNA purification columns. Briefly, confluent BSC40 cells or KO3 cells ($1x10^7$ cells in 100 mm diameter dishes) were infected with wt or mutant virus at an MOI of 15 at 31^0C (permissive temperature) or 40^0C (nonpermissive temperature). At various times after infection, total cellular RNA was purified using RNeasy Total RNA purification columns as described (Quiagen, Inc., Chatsworth, CA). Briefly, cells were harvested in 600 ul RLT Lysis Buffer and triturated 5 times with a narrow gauge hypodermic needle. Lysed cells were mixed with 600 ul 70% ETOH and loaded on a RNeasy spin column. The column was washed once with 700 ul RW1 wash buffer, twice with 500 ul RPE wash buffer. The RNA was eluted from column with DEPC-treated H_2O (DEPC-H_2O) and quantitated by measuring absorbance at 260 nm.

Northern Blot Analysis

Purified RNA was denatured in formamide and electrophoresed through 1.2% formaldehyde agarose gels as described in Sambrook et al., 1989. The RNAs were

transferred to GeneScreen membrane (New England Nuclear), prehybridized and hybridized with uniformly labeled antisense riboprobes as described by the manufacturer.

***In vitro* Transcription**

Infected cell extracts for transcription were prepared by lysolethicin permeabilization of infected cell monolayers as described previously (57). Briefly, transcription was assayed by incubation of extracts with DNA templates containing vaccinia virus promoter-driven G-less cassettes in the presence of ATP, UTP, [α-^{32}P]CTP, and 3'-O-methyl-GTP at 30^{0}C for 30 min. Labeled RNA products were purified and analyzed by electrophoresis on 4% polyacrylamide urea gels.

RT-PCR Analysis

RNA was extracted from virus infected BSC40 cells and purified on RNeasy Total RNA purification columns (Quiagen, Inc., Chatsworth, CA) as described above. For RT-PCR, the eluted RNA was DNase treated and repurified as described in the Invitrogen RNA kit. Briefly, the RNA was incubated with RNase-free DNase I at 37^{0}C for 30 min. The reaction was then mixed with 450 *u*l of Binding buffer and 300 *u*l of isopropanol. The solution was transferred to a S.N.A.P.^{TM} Total RNA column, and spun for 1 min. The column was washed with RNA Wash Buffer twice, and the RNA was eluted with 125 *u*l of RNase-free water.

Primers were designed by reference to the genomic sequence of the Copenhagen strain of vaccinia virus and synthesized at the University of Florida ICBR DNA Synthesis

Core laboratory. The sequences for primers were: for experimental primers which extend from K2L to M1L, forward primer (RIC266) 5' GAATGCAAAGATAGATGTCG 3' (NT 29377-29358), reverse primer (RIC267) 5' GACTTCATCATCTGTTCCCG 3' (NT 26912-26931); for control primers which amplify within the K2L region, forward primer (RIC245) 5' CGTGTTCAGTGTCTACCTATC 3' (NT 30285-30264), reverse primer (RIC268) 5' AACGACGCATTATCTGGA 3' (NT 29424-29441).

Purified DNA-free RNA (1 *u*g) was incubated at 42^0C for 1 hr with 200 units of M-MLV reverse transcriptase (Promega) in a 20 *u*l reaction containing 50 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM dNTPs, 25 pmol experimental reverse primer, 6 pmol control reverse primer, and 20 units RNasin. Control reactions lacking either reverse transcriptase or template RNA were performed. For PCR amplification, 2 *u*l of the 20 *u*l RT mixtures were added to 23 *u*l PCR mixture, containing: 1X PCR buffer (Promega), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 6 pmol each of experimental primers or 1.25 pmol each of control primers, 0.25 *u*Ci α-[^{32}P]-dCTP, and 1.25 units Taq polymerase (Promega). Cycling conditions (determined empirically) were: an initial denaturation step of 2 min at 94^0C, followed by 30 cycles of 94^0C for 30 sec, 51^0C for 30 sec, 72^0C for 3 minutes, followed by a final extension at 72^0C for 5 minutes. Seven *u*l of the reaction were loaded on 0.8% nondenaturing agarose gel. The gel was dried and the RT-PCR products were detected by autoradiography and quantified by analysis on a Molecular Dynamics phorsphorimager.

One Step Growth Experiment

One step growth experiments were performed as previously described. Cells were infected with wt or C*ts*23 virus at MOI of 6 to ensure that only one cycle of growth is measured. At various times post infection, virus was harvested and the yield from each time point was quantified by plaque titration assay.

Protein Pulse Labeling Analysis

Pulse-labeling of proteins in virus infected cells was done as described previously (58). Briefly, confluent monolayers of KO3 cells in 35 mm dishes were infected with wt or C*ts*23 at a MOI of 15 or mock infected. At various times post infection, cells were metabolically labeled with 10 μCi of Trans [^{35}S] methionine (ICN Bioochemical, Irvine, Calif.) in 0.5 ml of PBS for 15 min. Cells were lysed on the dishes by addition of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, and solubilized proteins were analyzed by SDS-PAGE using 10% separating gels. The gels were Coomassie blue stained, dried, and autoradiographed.

Results

Promiscuous Transcription Is Context Sensitive

The phenotype of infection with A18R mutants is characterized by promiscuous transcription, (originally called "aberrant transcription") (21). Promiscuous transcription is defined as transcription within regions of the genome, for example early genes D9R or

G2R, which are normally transcriptionally silent late during wt infection. Previous RNase protection and northern blot results are not consistent with two hypotheses that would account for promiscuous transcription: reinitiation at early promoters and promoter-independent random transcription throughout the genome (317). A compilation of the previous results obtained to date concerning transcription in A18R mutant infected cells also reveals that promiscuous transcription is "context sensitive" (21;317). Specifically, promiscuous transcription has been observed within the D9R and G2R genes, but not within the C11R and F17R genes. Interestingly, inspection of the vaccinia virus genetic map (Fig. 2-1) reveals that the C11R and F17R genes are unusual in that there are no other known promoters in the same transcriptional orientation within 4 kb upstream from either gene. By contrast, D9R and G2R each lie 3-5 kb downstream from intermediate or late promoters driving transcription of upstream genes. These observations provide further support for the hypothesis referred to below as the "readthrough hypothesis" that promiscuous transcription results from transcriptional readthrough from upstream intermediate or late gene promoters.

Promiscuous Transcription of the A18R Mutants in the Region from K2L to M1L

In order to test the readthrough hypothesis, we have focused attention on a region spanning the vaccinia genes M1L through K2L (Fig. 2-1). Previous northern blot analyses from other laboratories using exclusively early viral RNA show that M1L, M2L, K1L and K2L are each expressed early during infection. Furthermore, northern blot analysis by Smith et al (274) revealed transcription within the K2L region at late times.

Whether any of the four genes contains intermediate or late promoters is unknown. Preliminary data from our lab showed that the M2L gene was promiscuously transcribed. We hypothesize that promiscuous transcription in the M2L region results from readthrough transcription from an upstream intermediate or late promoter. As an initial test of this hypothesis, Dr. Simpson conducted northern blot analysis within this region using antisense riboprobes specific for M2L, K1L and K2L. I then extended the northern blot analysis to gene M1L. Northern blot analysis of M1L (Fig. 2-2) and M2L (317) shows that early M1L transcripts (1.6 kb) and early M2L transcripts (800 bp) appear at 1.5 hr post infection and decrease at late times in both wt and C*ts*23 infected BSC40 cells. The sizes of the early transcripts are consistent with previous results from other laboratories (273;285). At late times the M1L and M2L genes are transcriptionally silent in wt infections at either 31^0C or 40^0C. At late times in C*ts*23 infections at 31^0C the M1L gene is transcriptionally silent. Abundant transcription is observed in C*ts*23 infected cells at 40^0C after 6 hr post infection in both the M1L and M2L genes. Some of these transcripts have a shorter than normal chain length resulting from RNA degradation catalyzed by RNase L. These results show that both the M1L and M2L genes are promiscuously transcribed. The low level of transcription observed at 31^0C at late times in C*ts*23 infected cells within the M2L gene may indicate that the A18R gene product is not fully active at permissive temperatures. Nevertheless, promiscuous transcription observed at 31^0C in C*ts*23 infected cells is not sufficiently robust to either affect the M1L gene or activate RNase L. Northern blot analysis of the K1L gene (317) reveals a 1.1 kb early transcript, consistent with published experiments (273). Transcription is detected at

late times in both wt and C*ts*23 infections at both temperatures. Some of the late transcripts in C*ts*23 infected cells at 40^0C are shorter than normal, resulting from RNase L catalyzed RNA degradation. Northern blot analysis of the K2L gene (317) reveals two barely detectable early transcripts of 1.5 kb and 2.1 kb, consistent with published experiments (274). At late times the K2L gene is transcribed in both wt and C*ts*23 infections. Since intermediate and late vaccinia RNAs are normally heterogeneous in size and may read through into downstream genes, we cannot determine the origin of any of the late transcripts detected in this region from northern blot analysis alone. Nevertheless if the readthrough hypothesis is correct, we predict that promiscuous transcription of the M1L and M2L genes results from readthrough transcription originating from K1L, K2L, K3L or K4L. Detailed analysis of transcription initiation is required to further investigate this hypothesis.

Characterization of Gene Class in the Region from M1L to K2L

In order to further characterize the gene class in this region, we used RNase protection, combined with hydroxyurea (HU) and cycloheximide (CHX) "drug swap assay" to determine the kinetic class of each gene in this region. Published S1 nuclease analysis shows that M1L contains an early promoter, consistent with northern analysis (285). The published S1 nuclease analysis of the M1L gene also indicates the presence of a weak late promoter. This late promoter must be very weak, since late transcription occurs at only a very low level within the M1L gene, as revealed by northern analysis. We did not attempt to analyze transcription of the M1L gene further. RNase protection

analysis on RNA purified from cells treated with a "drug swap" protocol was designed to distinguish intermediate from late transcription. Vaccinia early, intermediate, and late RNA can be distinguished by analysis of RNA from cells infected in the presence of the DNA replication inhibitor hydroxyurea (HU), followed by a shift to drug-free medium or medium supplemented with the protein synthesis inhibitor cycloheximide (CHX) (16). Since intermediate and late gene transcription are coupled to DNA replication, only early genes are expressed in the presence of HU. Early gene expression includes synthesis of intermediate transcription factors, thus intermediate and ultimately late transcription proceeds and early gene expression is shut off when the HU block is removed. If CHX is added at the time HU is removed, early transcription continues and additional transcription is limited to intermediate genes since synthesis of late transcription factors encoded by intermediate genes is inhibited. Consistent with northern blot analysis, RNase protection analysis and "drug swap assay" showed M2L, K1L and K2L contain early promoters. The data showed further that neither M2L nor K1L contains a postreplicative promoter, and therefore that any intermediate or late transcription through these genes represents readthrough from upstream genes. Lastly, the results showed that K2L contains a postreplicative promoter and is also transcribed by readthrough from an upstream postreplicative promoter (317).

To confirm that K2L and K4L contain intermediate promoters, we analyzed these promoters in an *in vitro* transcription assay. In this assay, extracts from vaccinia virus infected, hydroxyurea treated cells are used to transcribe a template containing an intermediate promoter fused to a G-less cassette. HU treatment prevents intermediate

and late gene transcription and therefore prevents synthesis of late and early viral transcription factors (57). Thus HU treated extracts contain only early gene products, including intermediate gene transcription factors, and they are capable of initiating transcription at intermediate but not early or late vaccinia promoters. We cloned each of the candidate K2L and K4L promoter sequences upstream of the G-less cassette in pC_2AT19. Results of *in vitro* transcription directed by these templates are shown in Fig. 2-3. Control experiments with standard early (pVGFG DNA, C11R promoter), intermediate (pG8G DNA, G8R promoter) and late (pCFW10 DNA, F17R promoter) templates (lanes 1 to 3 and 9 to 11), show that early, intermediate and late transcripts are detected with the extracts made in the absence of HU, while only intermediate transcripts are detected with extracts made in the presence of HU. In addition, the intermediate template is transcribed better in the +HU extracts (lanes 2 and 10). *In vitro* transcription with the pK2 template shows that, like the G8R control, the K2L promoter is transcribed more efficiently with +HU extracts (lane 4) than with -HU extracts (lane 12), confirming that the K2L promoter has intermediate character. *In vitro* transcription with the pK4 template shows that it is transcribed very weakly in a high concentration of +HU extract (lanes 5 to 8), suggesting that K4L contains a weak intermediate promoter. In summary, both the K2L and the K4L promoters have intermediate characteristics in the *in vitro* transcription analysis.

The results from detailed transcription analysis of the M1L through K2L region indicate that M1L, M2L and K1L are exclusively early genes, and that K2L contains a compound early/intermediate promoter. In addition, the K4L promoter also has

intermediate character. Furthermore, in a wt infection, intermediate transcription reads through from upstream genes into both the K1L and K2L genes. Most of the readthrough into K1L probably derives from the K2L intermediate promoter, while readthrough into the K2L gene probably arises from the K4L promoter. We hypothesize that during an A18R mutant infection, transcription initiated from the K2L intermediate promoter reads through into the downstream early M1L and M2L genes, resulting in the promiscuous transcription phenotype.

RT-PCR Analysis of the Readthrough Transcription from K2L to M1L

We hypothesized that in an A18R mutant infection, more readthrough transcription from a candidate intermediate K2L promoter into downstream early genes M2L and M1L would be detected. We used RT-PCR as a direct measure for transcription which originates in K2L and extends into M1L or M2L. Analysis of the size of nascent transcripts in A18R mutant infections is complicated by the fact that promiscuous transcription induces RNase L catalyzed RNA breakdown. However, intermediate gene transcription begins at 3 hr post infection whereas RNA degradation is not evident until 7.5 hr post infection (data not shown) and reference (21). We, therefore, attempted to measure readthrough transcription by RT-PCR during the interval between the initiation of intermediate transcription and induction of RNase L (Fig. 2-4A). RNA was extracted from BSC40 cells infected with wt or C*ts*23 at various times post infection, and analyzed by RT-PCR using two sets of primers simultaneously. The experimental primer set amplified a 2466-nt readthrough transcript (E:2466) extending from K2L to

M1L, whereas the control primer set amplified an 862-nt transcript from within the K2L gene. Both transcripts were quantified by phosphorimager analysis and the ratio of the readthrough transcripts to the control transcripts (E:2466/C:862) was determined (Fig. 2-4B). As expected, the 862 nt control transcript appears early after infection, persists throughout the experiment, and is present in similar amounts regardless of the virus or temperature used. Surprisingly, the long 2466-nt transcript is observed in wt infected cells at 31^0C (lanes 1 to 7), even though the 2-5A pathway is not induced at this temperature. At 40^0C these readthrough transcripts are reduced in abundance in the wt infection (lanes 8 to 14). These results indicate that the incubation temperature effects the steady state level of readthrough transcription and also activation of the 2-5A pathway in wt infected cells. The amount of readthrough transcription observed in C*ts*23 infected cells at 31^0C (lanes 15 to 21) is slightly increased relative to the wt infection at 31^0C (lanes 1 to 7), consistent with the previous suggestion that the C*ts*23 mutant is slightly defective even under permissive conditions. Most importantly, at 40^0C after induction of intermediate transcription but before induction of the 2-5A pathway, the transcripts which extend from K2L into M1L are much more abundant in the C*ts*23 infection (lanes 23 to 26) compared to the wt infection (lanes 8 to 12). At later times, the C*ts*23 readthrough transcripts disappear (lanes 27 - 28) due to activation of 2-5A pathway. (The 862 nt control transcript is presumably small enough to be a poor target for RNase L *in vivo*.) In summary, at 40^0C, more readthrough transcription is detected in the C*ts*23 infection preceding RNA breakdown, which supports the hypothesis that the A18R mutation causes extended readthrough transcription from intermediate promoters.

Readthrough Transcription in an RNase L Knockout Cell Line (KO3)

In order to prove conclusively that the A18R mutation causes readthrough transcription, we investigated the A18R mutant phenotype in mouse KO3 cells which are derived from an RNase L knockout mouse and which therefore lack RNase L activity (326). Preliminary experiments demonstrated that infection of KO3 cells with C*ts*23 at 40^0C does not cause rRNA breakdown (data not shown). Interestingly, C*ts*23 infection of normal control cells from the parental mouse at 40^0C also revealed no rRNA breakdown, and therefore differences observed during vaccinia infection of KO3 cells compared to BSC40 cells cannot be attributed solely to a lack of RNase L. Nevertheless, KO3 cells provide a method for analyzing the A18R mutant phenotype in the absence of RNA degradation. In KO3 cells we expect to observe abnormally long transcripts in A18R mutant infections in regions where promiscuous transcription occurs. Northern blot analysis using an M2L riboprobe was performed using RNAs extracted at various times from virus infected KO3 (Fig. 2-5). The results show that the discrete 800 nt M2L early RNA is expressed in a similar profile in C*ts*23 and wt infected KO3 cells at both permissive and nonpermissive temperatures. In addition, larger heterogeneous readthrough transcripts appear at late times in both C*ts*23 and wt infected KO3 cells at both temperatures. Most importantly, the readthrough transcripts observed in C*ts*23 infected KO3 cells at 40^0C include a large population of transcripts longer than 4.4 kb which are not observed under any other condition of infection. This result is consistent with readthrough transcription from upstream genes into the M2L region in A18R mutant

infected KO3 cells. Northern blot analysis using M1L, K2L, D9R and G2R riboprobes show the similar results (data not shown).

A18R Mutant Phenotype in KO3 Cells

KO3 cells provide an opportunity to study whether the A18R gene is essential under circumstances where the 2-5A pathway is not activated. C*ts*23 is temperature sensitive on KO3 cells in a plaque assay (data not shown). A one step growth experiment was done to quantify the growth of wt and C*ts*23 on KO3 cells (Fig. 2-6). Wild type virus grown at 31^0C or 40^0C shows a burst size between 80 and 800 plaque-forming units per cell with maximum yield occurring after 48 hr post infection. Growth of C*ts*23 at 31^0C is identical to wt. C*ts*23 does not grow on KO3 cells at 40^0C. This result shows that the A18R gene is essential even in the absence of 2-5A pathway induction.

Protein pulse labeling was used to compare patterns of protein synthesis of wt and C*ts*23 infected BSC40 cells (Fig. 2-7). BSC40 cells were infected with wt or C*ts*23; and at various times post infection, cells were pulse labeled with [^{35}S] methionine, and the labeled proteins were analyzed by SDS-polyacylamide gel electrophoresis and autoradiography. The profiles of protein synthesis observed in wt infected cells at 31^0C and 40^0C, or in C*ts*23 infected cells at 31^0C are identical. Specifically, early viral proteins are expressed concomitant with shutoff of host cell protein synthesis, followed by expression of intermediate and late viral proteins which persists throughout the experiment. In a C*ts*23 infection at the nonpermissive temperature, host shutoff and early viral protein synthesis appear normal, and late protein synthesis is initiated at a normal

time, but synthesis of late proteins is abolished at late times. The same assay was done to examine the pattern of gene expression in wt and C*ts*23 infected KO3 cells (Fig. 2-8). Protein synthesis of wt and C*ts*23 at 31^0C is identical and representative of the normal pattern of vaccinia gene expression observed on BSC40 cells. Late protein synthesis is initiated at a normal time, but synthesis of late proteins is reduced in amount throughout the duration of the experiment in a C*ts*23 infection at the nonpermissive temperature. The defective late protein synthesis phenotype is different from the abortive late phenotype observed in infection of BSC40 cells (Fig. 2-7). The abortive late protein synthesis in *Cts*23 infected BSC40 cell results from the shutoff of the protein synthesis induced by the activation of 2-5 A pathway. In the absence of the activation of 2-5 A pathway, late viral protein synthesis on KO3 cells is decreased rather than abolished. Thus the A18R mutant virus is defective in late protein synthesis on KO3 cells.

The defective late protein synthesis phenotype seen in C*ts*23 infected KO3 cells could be due to a deficiency in viral mRNA metabolism. To test this hypothesis, we used northern blot analysis to determine the kinetics of mRNA synthesis, and the size and quantity of the steady state mRNAs synthesized. Total cellular RNA extracted from KO3 cells infected with wt or C*ts*23 was hybridized with antisense riboprobes specific for early (C11R), intermediate (G8R) or late (F17R) genes (Fig. 2-9). Early C11R mRNAs in wt and C*ts*23 infected cells are expressed at the same times post infection, in the same quantities, and appear as discrete bands, regardless of the incubation temperature. Intermediate G8R mRNAs in wt and C*ts*23 infected KO3 cells are expressed at the same time post infection, in similar quantities, and appear as smears diagnostic of the expected

3' end heterogeneity. In the C*ts*23 infection done at 40^0C, there is an increase in intermediate transcripts larger than 4.4 kb, consistent with northern analysis of the M2L gene (Fig. 2-5). In C*ts*23 infected cells, late F17R mRNA synthesis is initiated at the appropriate time post infection, but the quantities of late mRNA is a little reduced at 31^0C and significantly decreased at 40^0C. In summary, synthesis of steady state late mRNA in A18R mutant infected KO3 cells is reduced in quantity, consistent with the defective late protein synthesis phenotype described above.

In summary, our analysis indicates that transcription originates from intermediate promoters and continues readthrough transcription into downstream early genes in an A18R mutant infection. This readthrough transcription affects subsequent gene expression.

Discussion

The experiments described here were done in an attempt to refine our understanding of the effects of the vaccinia virus A18R gene on postreplicative viral transcription. Previous research had shown that mutations in the A18R gene cause promiscuous transcription, that is, transcription from regions of the genome which are normally transcriptionally silent late during infection. Previous results eliminate two possible explanations for promiscuous transcription: (1) the early VGF, M2L and K1L promoters do not reactivate at late times post infection and (2) random transcription throughout the genome does not occur. Our detailed analysis of transcription within the

M1L - K2L region of the viral genome provides positive support for the only remaining explanation for promiscuous transcription. Specifically, both RT-PCR analysis conducted in virus infected BSC40 cells and northern analysis conducted in RNase L knockout KO3 cells show that in A18R mutant infections transcription initiated from the K2L intermediate promoter yields longer than normal transcripts which read through into the downstream early M1L gene. In summary, these results show that late during a wild type virus infection, the A18R gene product limits elongation by the viral RNA polymerase, and thus has the properties of a negative transcription elongation factor.

In the course of our characterization of the A18R mutant, we have carried out a detailed transcription analysis of gene class in the region spanning the M1L and K2L genes which both confirms and extends previous analysis of individual genes within this region. Perhaps the most important outcome of this transcription analysis is the discovery of two new intermediate genes, K2L and K4L. The discovery of two new intermediate genes, K2L and K4L, provides additional insight into intermediate promoter structure. Published analysis of the five known intermediate genes (15;120;299) shows that intermediate promoters are approximately 30 nt in length and contain two critical regions, an upstream 14-bp core element that is A/T rich, separated by 10 or 11 bp from a 4-bp initiator element which contains the sequence TAAA. Our previous RNase protection assay shows that K2L has intermediate promoter activity *in vivo*, and an mRNA 5' end with characteristics of an intermediate RNA maps within 10 nt upstream of the K2L translation initiation codon. We have shown here that the 38 nt sequence upstream from both the K2L and K4L translation initiation codons have intermediate

promoter activity *in vitro*. Inspection of the K2L and K4L upstream regions reveals sequence which matches precisely the initiator TAAA, and which closely approximates the A/T rich core (Fig. 2-10). Closer inspection of these sequences, allowing for inclusion of 10 or 11 bp in the spacer region, reveals deviations from a proposed intermediate consensus sequence which could both increase and decrease promoter activity (15). For example, both promoters contain a potentially inhibitory deviation from a consensus AAA in the –17 to -19 region, while both contain potentially stimulatory deviations from the G8R sequence in the -20 and -23 regions.

The discovery that K2L and K4L are intermediate genes provides some additional insight into the functional organization of the intermediate gene class. The five previously characterized intermediate genes, three late transcription factors (136), a virion RNA helicase (265) and a single stranded DNA binding protein (244), all map within the central conserved region of the virus genome and are all implicated in nucleic acid metabolism. In contrast, while the function of K4L is unknown, K2L encodes SPI3, a serine protease inhibitor homolog which plays a role in virus induced cell fusion (292), and both K2L and K4L map in the variable left terminus of the genome. Importantly, any vaccinia gene which has been previously classified as a late gene and not specifically tested for intermediate gene activity is potentially an intermediate gene. In summary, our observations suggest that the intermediate gene class may in fact be relatively large and include genes with a wide variety of functions.

Our phenotypic analysis of the A18R mutant infection of KO3 cells provides fresh insight into the primary consequences of readthrough transcription on the viral

infection. Interpretation of prior phenotypic analysis of A18R mutant infections, done exclusively on BSC40 cells, was complicated by the fact that readthrough transcription from converging promoters causes an accumulation of dsRNA, which triggers the 2-5A pathway, which in turn activates RNase L and causes a global degradation of mRNA and rRNA and a cessation of protein synthesis (21;53;220). Thus it was unclear whether in the absence of RNase L activity, the A18R gene would be essential and whether readthrough transcription would have deleterious effects on the infection. Significantly, we have found that, in RNase L knockout KO3 cells, the A18R mutant virus is temperature sensitive with respect to virus growth, and that steady state viral late mRNAs and late viral proteins are present in reduced amounts. Several possible explanations exist for the observed defect in late gene expression on KO3 cells. First, it is formally possible that the A18R gene product is directly involved in the initiation of late viral transcription. We feel this possibility is unlikely since *in vitro* experiments from our lab (data not shown) and from other labs (144;314) have failed to demonstrate any role of A18R in initiation of late viral transcription. Second, readthrough transcription from converging intermediate promoters should still cause accumulation of dsRNA in KO3 cells, therefore the dsRNA dependent protein kinase (PKR) pathway (155) may be activated at intermediate times. Thus, inhibition of synthesis of late viral transcription factors could in turn cause defective synthesis of late mRNAs. Third, it is possible that formation of dsRNA results in interference with translation of late transcription factors from intermediate mRNAs, also impacting on late mRNA synthesis. Fourth, readthrough transcription could result in direct interference with initiation of transcription from

downstream genes, a phenomenon previously documented in studies of transcription in mammalian cells (82). Interference of pol II promoters was first described in retroviruses, notably the avian leukosis virus (ALV), where initiation at the 3' long terminal repeat (LTR) is repressed by the transcriptionally active 5' LTR (67). Subsequently, two closely spaced α-globin genes in an artificial gene construct were also shown to interfere with each other (229). Furthermore, a single poly (A) signal or transcription pause sites or a combined element of a poly (A) signal followed by a pause site is able to prevent transcription interference. Unfortunately, the decrease in late mRNA synthesis in A18R mutant infected cells has so far made it difficult to determine whether the A18R mutation affects readthrough transcription from late as well as intermediate promoters. In any case, the phenotypic analysis of A18R mutant infections on KO3 cells emphasizes the importance of restricting readthrough transcription from intermediate promoters during a normal vaccinia infection.

In both eukaryotic and prokaryotic systems, a variety of negative transcription elongation factors have been identified. Virtually all of these factors have termination factor activity, defined experimentally as the release of nascent transcripts from a ternary elongation complex, and many are helicases and/or nucleic acid dependent ATPases. The *E. coli* factor Rho is the most extensively studied termination factor (131;304). Rho is an RNA dependent ATPase and an RNA-DNA helicase which is thought to bind nascent RNA, and translocate in the 5' to 3' direction along the RNA in an ATP dependent fashion, finally causing the dissociation of the ternary elongation complex by unknown mechanisms. Drosophila factor 2, a double stranded DNA dependent ATPase which

lacks detectable helicase activity (318) can cause the release of RNA polymerase II transcripts in an ATP-dependent manner (319). Recently it has been shown that in vaccinia virus, the ATP dependent step in early transcription termination is catalyzed by a single stranded DNA dependent ATPase, the product of gene D11L, which also lacks detectable helicase activity (73). Thus it is clear that transcription termination in several systems requires the participation of a factor which can bind single or double stranded RNA or DNA and hydrolyze ATP. We have shown here that the A18R protein is a negative transcription elongation factor. We have shown previously that the A18R protein, a member of the DExH helicase superfamily II (143), possesses both DNA dependent ATPase and DNA helicase activities. The ATPase activity of A18R is stimulated by both single and double stranded DNA but not by RNA (22). The helicase activity is restricted exclusively to DNA:DNA hybrids, it is only capable of separating hybrids containing less than 25 bp, and it displays 3' to 5' directionality (272). Thus based on prior biochemical analysis of the A18R protein, on the A18R mutant analysis presented here, and by analogy with other known transcription termination factors, we propose that A18R serves as a termination factor for intermediate and perhaps late transcription *in vivo*.

Our experiments imply that 3' end formation during postreplicative vaccinia transcription is a factor mediated event, but provide no information about potential cis acting elements in either RNA or DNA that might be required for termination. In fact, termination of vaccinia postreplicative transcription resembles termination of transcription in eukaryotic cells in that it occurs at a large number of sites, generating

extreme 3' end heterogeneity (109;164;229). Thus if specific nucleic acid sequences or structures mediate postreplicative vaccinia transcription termination, these elements must be both abundant and inefficient.

Additional biochemical experiments (29) and genetic experiments (Chapter 4) suggest that the A18R protein does not act alone but rather as part of a larger complex containing the viral RNA polymerase, the viral transcription factors G2R, J3R and perhaps other factors as well. Biochemical experiments designed to elucidate the precise activities of A18R, G2R and H5R in an elongating RNA polymerase complex are underway.

Fig. 2-1. Arrangement of coding regions within the vaccinia virus genome. This figure was taken from (99). The vaccinia virus genome is depicted as a series of 20-kb fragments. The scale is numbered in kb from the left terminus. The sizes and orientations of rightward and leftward ORFs are indicated by arrows above and below the line respectively. Genes are named according to the Hind III fragment in which they are located, the order (left to right except for the C fragment) within the fragment, and the orientation of the gene.

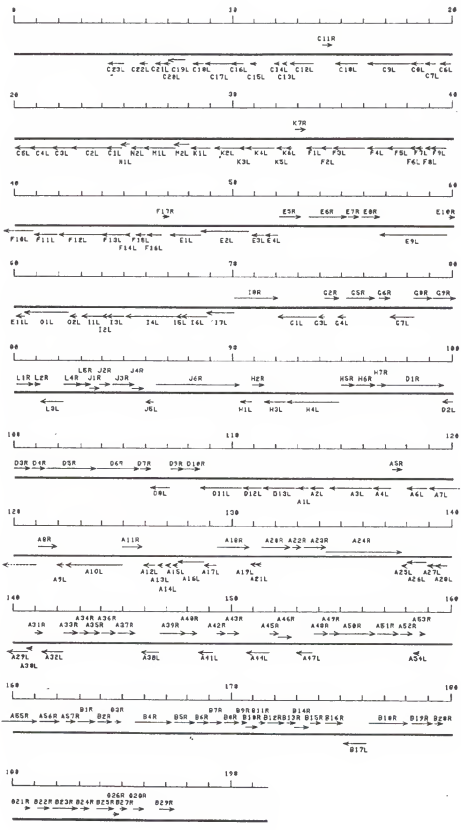

C11R
C22L C22L C21L C19L C18L C16L C14L C12L C18L C9L C6L C6L
C28L C17L C15L C13L C7L
K7R
C6L C4L C3L C2L C1L M2L M1L P2L R1L K2L K4L K6L F1L F3L F4L F5L F7L F6L
M1L K3L K5L F2L F6L F8L
F13R E5R E6R E7R E8R E10R
F10L F11L F12L F13L F15L F14L F16L E1L E2L E3L E4L E9L
I8R G2R G5R G6R G8R G9R
E11L O1L O2L I1L I2L I4L I6L I6L I7L C1L C3L C4L C7L
I2L
L1R L2R L4R J1R J3R J6R H2R H5R H6R H7R D1R
L2R J2R J4R
L3L J6L H1L H3L H4L D2L
D3R D4R D5R D6R D7R D9R D10R A5R
D8L D11L D12L D13L A2L A3L A4L A6L A7L
A1L
A8R A11R A16R A23R A22R A23R A24R
A9L A10L A12L A15L A17L A17L A22L A27L
A13L A16L A21L A26L A28L
A14L
A31R A33R A34R A36R A37R A39R A40R A43R A42R A44R A45R A49R A48R A50R A51R A52R A53R
A27L A32L A38L A41L A44L A47L A54L
A39L
B1R B3R B7R B9R B11R B14R
A55R A56R A57R B2R B4R B5R B6R B10R B12R B13R B15R B16R B18R B19R B20R
B17L
B21R B22R B23R B24R B25R B27R B29R
B26R B28R

Fig. 2-2. Promiscuous transcription in the M1L region in C*ts*23-infected BSC40 cells. BSC40 cells were infected with wt or C*ts*23 virus at an MOI of 15 and incubated at 31 or 40^0C. Total RNA was extracted from infected cells at various times post infection indicated in hr above the lanes. RNA was fractionated on formaldehyde-agarose gels, transferred to GeneScreen membranes, and probed with uniformly labeled antisense RNA riboprobes specific for the M1L gene. Lane M contains uninfected cell RNA. Sizes are denoted at the left in kilobases (kb). The cartoon represents the M1L to K2L region. Gene names are indicated above arrows representing the open reading frames. The M1L probe used is shown in the cartoon. An interpretation of the transcriptional analysis, showing extended readthrough transcription from the K2L promoter into the M1L gene in a C*ts*23 infection.

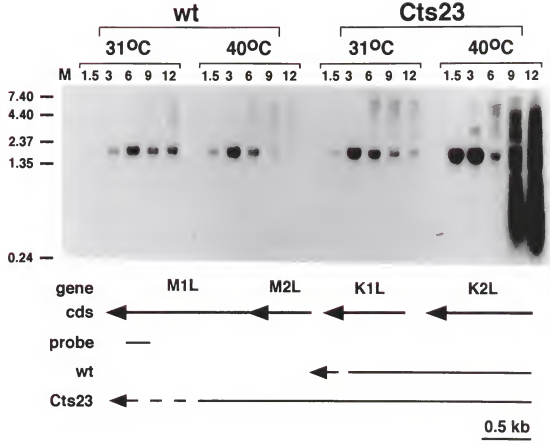

wt
Cts23
31°C
40°C
31°C
40°C
M 1.5 3 6 9 12 1.5 3 6 9 12 1.5 3 6 9 12 1.5 3 6 9 12
7.40
4.40
2.37
1.35
0.24
gene
M1L
M2L
K1L
K2L
cds
probe
wt
Cts23
0.5 kb

Fig. 2-3. In vitro transcription of templates containing K2L and K4L promoter sequences. Transcription-competent extracts were made from cells infected with wt virus in the presence of 10 mM hydroxyurea (+HU) or in the absence of drug (-HU). Transcription was done using 10 ul (lanes 1 to 5 and 9 to 13) or 15 ul (lanes 6 to 8 and 14 to 16) of extract. Reactions contained 10, 30 or 50 ug of pVGFG (early), pG8G (internediate), pCFW10 (late), pK2, or pK4 DNA per ml, as indicated. Reaction products were separated on a 7% polyacrylamide gel, which was dried and autoradiographed.

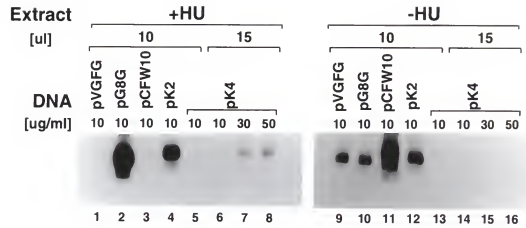
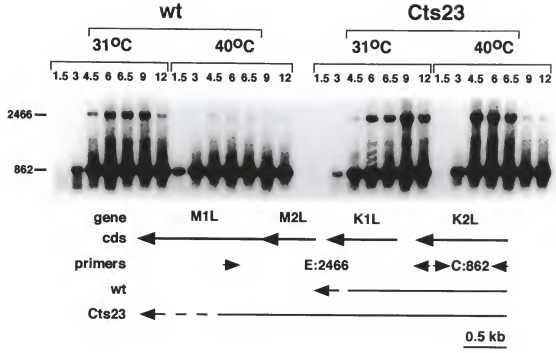

Extract
+HU
-HU
[ul]
10
15
10
15
DNA
pVGFG
pG8G
pCFW10
pK2
pK4
pVGFG
pG8G
pCFW10
pK2
pK4
[ug/ml]
10 10 10 10 10 10 30 50
10 10 10 10 10 10 30 50
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16

Fig.2-4. Readthrough transcription of the M1L gene in C*ts*23-infected BSC40 cells detected by RT-PCR analysis. The total RNA was purified from infected BSC40 cells as described in Fig. 2-3. RNA was DNase treated and analyzed by RT-PCR using primers extending from M1L into K2L, generating a 2466 nt long product (E:2466 in cartoon). Internal control primers measured a K2L RNA of 862 nt (C:862 in cartoon). The cartoon represents the interpretation of transcription analysis in the region of M1L to K2L. RT-PCR signals were quantified by phosphorimage analysis, and the ratio of the E:2466 signal to the C:862 signal was plotted as a function of time post infection.


wt
Cts23
31°C
40°C
31°C
40°C
1.5 3 4.5 6 6.5 9 12 1.5 3 4.5 6 6.5 9 12
1.5 3 4.5 6 6.5 9 12 1.5 3 4.5 6 6.5 9 12
2466
862
gene
M1L
M2L
K1L
K2L
cds
primers
E:2466
C:862
wt
Cts23
0.5 kb

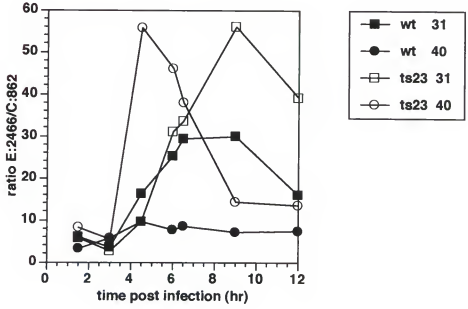

ratio E:2466/C:862
time post infection (hr)
0
10
20
30
40
50
60
0
2
4
6
8
10
12
wt 31
wt 40
ts23 31
ts23 40

Fig. 2-5. Readthrough transcription of the M2L gene in C*ts*23-infected KO3 (RNase L$^-$) cells. The total RNA was purified from infected KO3 cells as described in Fig. 2-3. RNA was analyzed by northern blot analysis using a uniformly labeled antisense riboprobe specific for M2L gene (Fig. 2-1). Sizes are denoted at the left in kb. The cartoon represents the M1L to K2L region as described in the Fig. 2-2 legend.

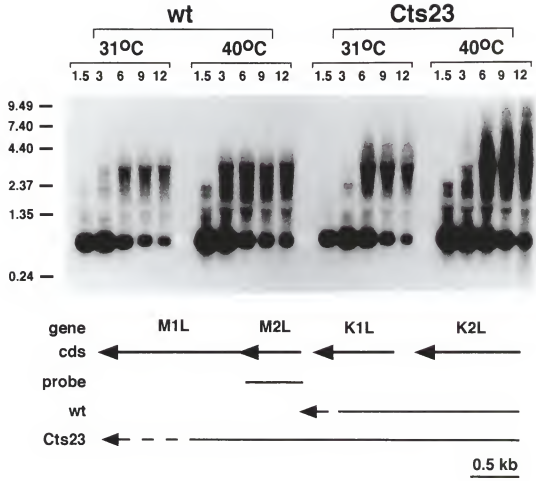

wt
Cts23
31°C
40°C
31°C
40°C
1.5 3 6 9 12
1.5 3 6 9 12
1.5 3 6 9 12
1.5 3 6 9 12
9.49
7.40
4.40
2.37
1.35
0.24
gene
M1L
M2L
K1L
K2L
cds
probe
wt
Cts23
0.5 kb

Fig. 2-6. One-step growth of wt and C*ts*23 in KO3 cells. KO3 cells were infected at an MOI of 6 with either wt or C*ts*23, and incubated at 31 or 40^0C. Samples were taken at various times post infection and virus yields were determined by plaque titration at 31^0C.

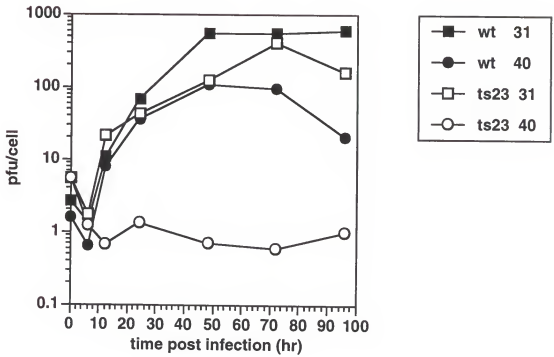

1000
100
10
1
0.1
pfu/cell
0 10 20 30 40 50 60 70 80 90 100
time post infection (hr)
wt 31
wt 40
ts23 31
ts23 40

Fig. 2-7. Protein synthesis in wt- and C*ts*23-infected BSC40 cells. BSC40 cells were infected with wt or C*ts*23 at an MOI of 15, incubated at 31 or 40^0C, and pulse labeled for 15 min with Trans [^{35}S] Methionine at the times post infection indicated above the lanes in hours. Lanes M, mock infection. Labeled proteins were electrophoresed on SDS-10% polyacrylamide gels and autoradiographed.

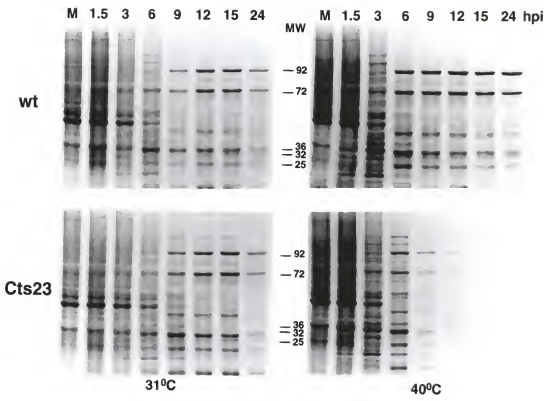

M 1.5 3 6 9 12 15 24
MW
M 1.5 3 6 9 12 15 24 hpi
wt
Cts23
92
72
36
32
25
31°C
40°C

Fig. 2-8. Protein synthesis in wt- and C*ts*23-infected KO3 cells. KO3 cells were infected with wt or C*ts*23 at an MOI of 15, incubated at 31 or 40^0C, and pulse labeled for 15 min with Trans [^{35}S] Methionine at the times post infection indicated above the lanes in hours. Lanes M, mock infection. Labeled proteins were electrophoresed on SDS-10% polyacrylamide gels and autoradiographed.

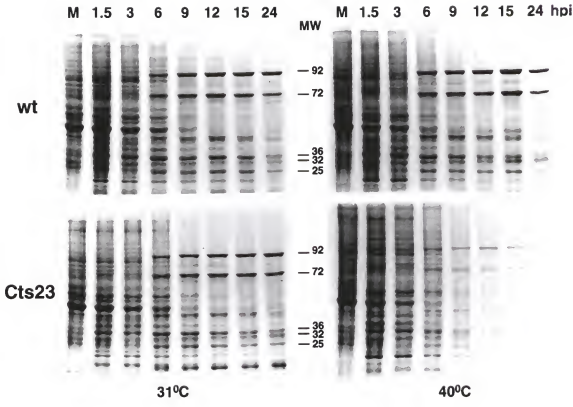

M 1.5 3 6 9 12 15 24
MW
M 1.5 3 6 9 12 15 24 hpi
wt
Cts23
92
72
36
32
25
31°C
40°C

Fig. 2-9. Northern blot analysis of RNA synthesized in wt- and C*ts*23-infected KO3 cells. The total RNA was purified from infected KO3 cells as described in Fig. 2-3. RNA was analyzed by northern blot analysis using uniformly labeled antisense RNA riboprobes specific for an early (E), intermediate (I), or late (L) gene. Lanes M contain uninfected cell RNA. Sizes are denoted at the left in kb.

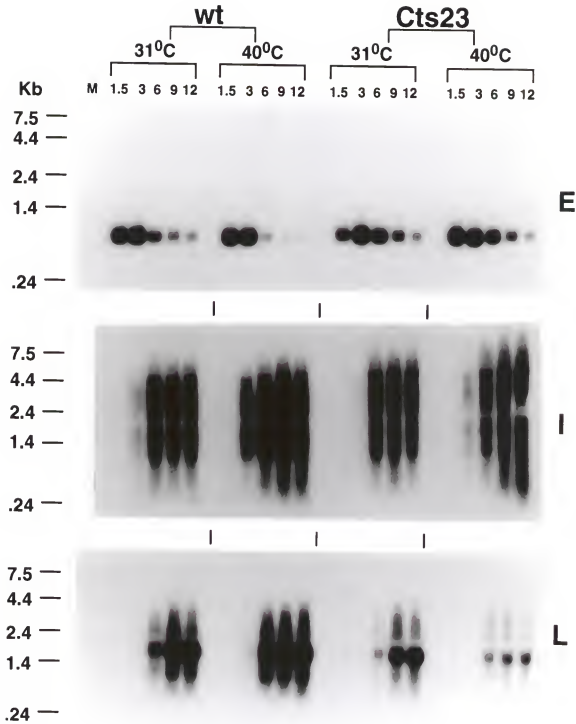
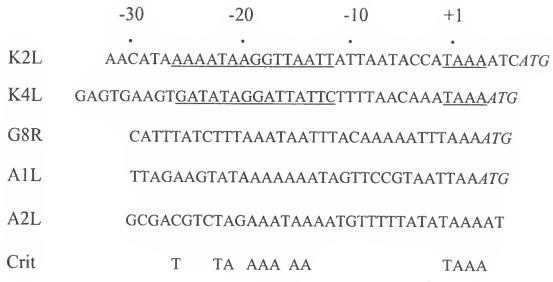

wt
Cts23
31⁰C
40⁰C
31⁰C
40⁰C
Kb
M 1.5 3 6 9 12 1.5 3 6 9 12 1.5 3 6 9 12 1.5 3 6 9 12
7.5
4.4
2.4
1.4
.24
E
I
L

Fig. 2-10. Intermediate gene upstream sequences. The upstream sequences from five intermediate genes are aligned relative to the critical TAAA initiator region. The G8R, A1L, A2L and critical (Crit) sequences are from Baldick et al. (15). The critical sequence represents nucleotides in the G8R promoter in which two of three possible substitutions decrease activity by greater than or equal to 75%. Putative core and initiator regions in the K2L and K4L sequence are underlined. Translation initiation ATGs are italicized.


-30
-20
-10
+1
K2L
AACATAAAAATAAGGTTAATTATTAATACCATAAAATC*ATG*
K4L
GAGTGAAGTGATATAGGATTATTCTTTTAACAAATAAA*ATG*
G8R
CATTTATCTTTAAATAATTTACAAAAATTTAAA*ATG*
A1L
TTAGAAGTATAAAAAAATAGTTCCGTAATTAA*ATG*
A2L
GCGACGTCTAGAAATAAAATGTTTTTATATAAAAT
Crit
T TA AAA AA
TAAA

CHAPTER 3
CHARACTERIZATION OF THE E3L AND A18R MUTANT PHENOTYPE IN THE RNASE L KNOCKOUT CELLS (KO3) AND RNASE L/PKR DOUBLE KNOCKOUT CELLS (DKO)

Introduction

Virus infection in host cells results in induced expression of interferon (IFN) and subsequent secretion of IFN to neighboring cells. This response is induced by synthesis of increased level of double-stranded RNA (dsRNA) in most virus-infected cells. IFN interacts with receptors on the neighboring cells and induces expression of several enzymes in those cells, including 2-5A synthetase and a protein kinase (PKR), which is also known as P68 kinase (Fig. 3-1). Activation of these enzymes is dependent on the presence of dsRNA. In the 2-5A pathway, activated 2-5A synthetase polymerizes short 2'-5' linked oligoadenylates (2-5A) from ATP. This 2-5A in turn activates a latent ribonuclease, RNase L, which is present in all animal cells. Activated RNase L can cleave viral and cellular RNAs (53). The 2-5A-dependent RNase L has nine ankyrinlike repeats at its amino terminus. The 2-5A binding domain, a GKT tripeptide, overlaps with some of these repeats. RNase L also has a cysteine-rich domain and a protein kinase

homology domain. The C-terminus of the protein contains the RNase activity which cleaves single-stranded RNA with moderate specificity (79). In the PKR pathway, PKR is also expressed constitutively at low levels in most cell types studied and PKR exists in an inactive form in uninfected cells. PKR activation by dsRNA results in autophosphorylation on several serine and threonine residues. The activated protein kinase phosphorylates α subunit of the eukaryotic translation initiation factor eIF-2α, which in turn shuts down of both viral and host protein synthesis (224). The induction of these two pathways in the neighboring cells will make the cells prepared for infection.

The discovery of apoptosis triggered by IFN-induced pathways has brought fresh insight into the biological antiviral and anticellular action of IFN. Esteban and coworkers have shown that the expression of IFN-induced dsRNA-dependent protein kinase in HeLa cells resulted in apoptosis (154). Recently, Williams and coworkers reported that the mouse embryo fibroblasts from PKR gene knockout mice were resistant to apoptosis in response to dsRNA. The 2-5A/RNase L system has also been shown to be involved in activation of apoptosis in mammalian cells (46;77;326). Apoptosis was triggered by overexpression of RNase L (46) and was enhanced by coexpression of 2-A synthetase and RNase L (77). A 2-fold decrease in apoptosis was observed *in vivo* in the thymuses and spleens of RNase L knockout mice. These knockout mice showed remarkably enlarged thymuses as a result of suppression of apoptosis. Thus, the 2-5A and protein kinase systems are likely to contribute to the antiviral activity of interferon by inducing apoptosis of infected cells.

Vaccinia virus replication measured by RNA synthesis, protein synthesis, and virus yield, has been shown to be relatively resistant to the antiviral effects of IFN in most cell lines tested. Vaccinia virus can even prevent the IFN-mediated inhibition of vesicular stomatitis virus and poliovirus replication in doubly infected cells (83). This IFN resistance is thought to be due to inhibition of either of the two IFN-induced, dsRNA-dependent antiviral pathways: the 2-5A pathway and the protein kinase PKR pathway. Two vaccinia virus early genes, K3L and E3L, appear to be involved in the interference phenomenon. The K3L gene product, pK3, is a homologue of the α subunit of eIF-2. It functions as a tightly binding pseudosubstrate to down-regulate PKR activity *in vitro* (25). Moreover, a pronounced sensitivity of protein synthesis to IFN was observed in K3L-deleted vaccinia virus infected cells (23). The vaccinia E3L gene product, pE3, corresponds to the activity found in vaccinia virus infected cells described as SKIF (specific kinase inhibitory factor). The vaccinia E3L gene encodes two dsRNA binding proteins, p25 and p20. These proteins prevent activation of PKR and RNase L, presumably by sequestering dsRNA, thereby making it unavailable to activate PKR and RNase L (48;243). The evidence that E3L can prevent activation of PKR pathway *in vitro* is provided by Jacobs and co-workers. They showed that (i) PKR activation is inhibited in the presence of extracts from interferon-treated and E3L- transfected cells; (ii) removal of E3L-encoded protein by anti-p25 antibody results in loss of kinase inhibitory activity (48). Furthermore, a vaccinia virus containing an E3L gene deletion (vp1080) is sensitive to the effects of IFN. In interferon-treated cells, rRNA cleavages were observed 7 hr after infection with an E3L-deleted vaccinia virus but not after a wt

infection (23). The vp1080 phenotype can be reversed by expression of the reovirus S4 gene encoding the dsRNA-binding protein σ3 (23). In conclusion, both *in vitro* and *in vivo* experiments show that E3L is necessary for the vaccinia virus IFN-resistant phenotype (48). Recently, the direct proof that E3L act as an inhibitor of 2-5A pathway was provided by Esteban and co-workers (243). They assayed for apoptosis induced by the infection of an E3L deletion vaccinia virus in cells derived from PKR knockout mice. Their data showed that expression of 2-5A synthetase and/or RNase L in the above system induced apoptosis, while co-expression of E3L and 2-5A synthetase completely prevented apoptosis induced by this enzyme. These data strongly suggest that vaccinia virus E3L serves as an inhibitor of the IFN-induced 2-5A synthetase enzyme.

E3L also performs a host range regulatory function, since specific deletion of E3L results in replication in certain cell types but not in others. Specifically, the vp1080 cannot replicate in HeLa cells but it exhibits nearly wt replication in rabbit kidney RK-13 cells (24) and in BHK cells. HeLa cells contain constitutively high levels of PKR activity, while RK-13 cells do not contain detectable PKR activity in the absence of IFN treatment, thus providing a possible explanation for the host range phenotype of vp1080. Replication of vp1080 in HeLa cells can be rescued by transient expression of a plasmid containing a gene coding for one of several dsRNA binding proteins, including the E3L gene (49). This suggests that a functional dsRNA binding protein is necessary to promote replication of vaccinia virus E3L deletion mutant in HeLa cells.

The A18R mutant C*ts*23 infection in BSC40 cells results in increased level of dsRNA which in turn triggers the activation of 2-5A pathway and the breakdown of both

viral and cellular mRNA (21). We took advantage of the RNase L knockout cells (KO3) to investigate the phenotype of *Cts*23 and showed that the phenotype of *Cts*23 is similar in KO3 cells and in parental WT1 cells which retain the RNase L activity. RNA degradation is not detected either in *Cts*23 infected WT1 cells or KO3 cells (Chapter 2). We were concerned that the difference of *Cts*23 phenotype between BSC40 and KO3 cells was not completely due to lack of RNase L. We therefore attempted to use another vaccinia virus, the vp1080, to confirm the nature of the mouse RNase L knockout cell line. Since the dsRNA binding protein E3L could sequester dsRNA and in turn prevent the activation of the RNase L pathway, dsRNA-binding protein may be nonessential in cell lines which lack RNase L. The plaque assay and one step growth experiments discussed here show that vp1080 virus grows better in knockout cells than in wt cells. We also conducted a phenotypic analysis of A18R mutant phenotype on cells established from an RNase L and PKR double knockout mouse (DKO). A18R mutant is defective in late viral gene expression and remains temperature sensitive in the absence of RNase L and PKR pathway activation.

Materials and Methods

Cells and Viruses

The continuous African green monkey kidney cell line BSC40 and conditions for cell culture have been previously described (58;59). WT1 and KO3 cells (kindly provided by Dr. Robert Silverman) are immortalized fibroblasts established from a wt

mouse and an RNase L knockout mouse (326), respectively. They were grown in Eagle's Modified Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Baby Hamster Kidney (BHK) cells were kindly provided by Dr. Bertram Jacobs in Arizona State University. They were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics. Wild-type vaccinia virus strain WR, the conditions for their growth, infection, and plaque titration have been described previously (58;59). The conditions for E3L deletion mutant virus vp1080 (24) growth, infection are described as below. Briefly, BHK cells were grown to confluence in 150 mm dishes. Cells were inoculated with vp1080 at MOI of 0.1 and incubated at 37^0C for 2 hrs. The inoculum was then removed and replaced with fresh medium and the infected cells were incubated at 37^0C for another 48 hrs. Cells were harvested and concentrated by centrifugation. The cell pellets were resuspended in PBS. The infected cells were frozen and thawed twice before the viruses were titered.

Plaque Assay

Confluent cells in 60 mm dishes were infected with serial dilutions of wt or E3L deletion mutant virus. After 1/2 hr to 2 hrs incubation, the inoculum was removed and 4 ml of Seakem LE agarose/DME were added per dish. In some instances, plaque assays were done under liquid medium instead of an agar overlay. After four days of incubation, dishes with agar overlay were stained with neutral red overnight, while dishes with liquid medium were stained with crystal violet for one hour.

One Step Growth Experiment

One step growth experiment was performed as previously described. Cells were infected with wt or C*ts*23 or vp1080 virus at MOI of 6 to ensure that only one cycle of growth is measured. At various times post infection, viruses were harvested and the yield from each time point was quantified by plaque titration assay on PKR KO cells.

Riboprobes

Riboprobes were synthesized as described (Promega Protocols and Applications Guide), using as a template for T7 RNA polymerase linearized plasmid DNA (pGEM-VGF, pGEM-30K, pGEM-11K). Plasmids pGEM-VGF, pGEM-30K and pGEM-11K were kindly provided by Dr. Bernard Moss (NIH).

Isolation of RNA

RNA was extracted from infected cells and purified by RNeasy Total RNA purification columns. Briefly, confluent DKO cells ($1x10^7$ cells in 100 mm diameter dishes) were infected with wt or mutant virus at an MOI of 15 at 31^0C (permissive temperature) or 40^0C (nonpermissive temperature). At various times after infection, total cellular RNA was purified using RNeasy Total RNA purification columns as described (Quiagen, Inc., Chatsworth, CA). The RNA was eluted from column with DEPC-treated H_2O and quantitated by measuring absorbance at 260 nm.

Northern Blot Analysis

Purified RNA was denatured in formamide and electrophoresed through 1.2% formaldehyde agarose gels as described in Sambrook et al., 1989. The RNAs were transferred to GeneScreen membrane (New England Nuclear), prehybridized and hybridized with uniformly labeled antisense riboprobes as described by the manufacturer.

Protein Pulse Labeling Analysis

Pulse-labeling of proteins in virus infected cells was done as described previously (58). Briefly, confluent monolayers of DKO cells in 35 mm dishes were infected with wt or C*ts*23 at a MOI of 15 or mock infected. At various times post infection, cells were metabolically labeled with 10 *u*Ci of Trans [^{35}S] methionine (ICN Bioochemical, Irvine, Calif.) in 0.5 ml of PBS for 15 min. Cells were lysed on the dishes by addition of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, and solubilized proteins were analyzed by SDS-PAGE using 10% separating gels. The gels were Coomassie blue stained, dried, and autoradiographed.

Results

Characterization of the Growth of vp1080 in WT1 and KO3 Cells

The A18R phenotype is similar in RNase L knockout KO3 cells and in parental WT1 cells which retain the RNase L activity (Chapter 2). Ribosomal RNAs are intact at late times post C*ts*23 infection in both cell lines. Readthrough transcripts from upstream

intermediate promoter have been detected in both KO3 and WT1 cells. It has been reported that the parental WT1 cells contains very low levels of RNase L and extremely low levels of 2-5A synthetase comparing to other cell lines detected (Silverman RH unpublished observation), which might explain this phenotype. However, we attempted to use another vaccinia virus, an E3L deletion mutant (vp1080), to confirm the nature of the mouse RNase L knockout cell line with respect to vaccinia virus infection.

Plaque assays were done to compare wt virus and vp1080 growth in KO3 cells, WT1 cells and BSC40 cells. The results indicate that wt virus forms plaques in all three cell lines with similar efficiency, while vp1080 can only form plaques in KO3 cells but not in WT1 cells and BSC40 cells (Fig. 3-2). However, the plaques formed in vp1080 infected-KO3 cells are tiny compared to wt infected-KO3 cells. Therefore, vp1080 displays an intermediate growth phenotype in KO3 cells.

A one step growth experiment was performed to measure the growth of vp1080 in these three cell lines (Fig. 3-3). BSC40, KO3 and WT1 Cells were infected with wt or vp1080 virus at MOI of 6 to ensure that only one cycle of growth is measured. At various times post infection, viruses were harvested and the yield from each time point was quantified by plaque titration assay on KO3 cells. The results show that wt viruses grow equally well in all cell lines, yielding an average of 80 plaque forming units per cell after 24 hr of infection. Vp1080 grows poorly in KO3 cells compared to wt infection, forming about 10 plaque forming units per cell after 24hr post infection. However, vp1080 does not grow at all in either WT1 cells or BSC40 cells. Thus, vp1080 grows better in KO3 than in WT1 cells, which provides us a way to differentiate these two cell

lines. This result is consistent with the intermediate growth phenotype of vp1080 in KO3 cells using plaque assay.

Characterization of the A18R Mutant Phenotype in DKO Cells

A18R mutant displays defective late phenotype in late viral gene expression and late protein synthesis in KO3 cells (Chapter 2). We hypothesized that the defective late phenotype could be due to the activation of PKR pathway triggered by the increased level of dsRNA. DKO cells provide an opportunity to study whether the A18R gene is essential under circumstances where both the 2-5A and PKR pathways are blocked. C*ts*23 is temperature sensitive on DKO cells in a plaque assay (data not shown). This result shows that the A18R gene is essential even in the absence of both pathways.

Protein pulse labeling studies were done to examine the pattern of gene expression in wt and C*ts*23 infected DKO cells (Fig. 3-4). DKO cells were infected with wt or C*ts*23 and at various times post infection cells were pulse labeled with [^{35}S] methionine, and the labeled proteins were analyzed by SDS-polyacylamide gel electrophoresis and autoradiography. The profiles of protein synthesis observed in wt infected cells at 31^{0}C and 40^{0}C, or in C*ts*23 infected cells at 31^{0}C are identical. Specifically, early viral proteins are expressed concomitant with shutoff of host cell protein synthesis, followed by expression of intermediate and late viral proteins which persists throughout the experiment. In a C*ts*23 infection at the nonpermissive temperature, host shutoff and early viral protein synthesis appear normal, and late protein synthesis is initiated at a normal time, but synthesis of late proteins is reduced in amount

throughout the duration of the experiment. The decrease in late protein synthesis is not as dramatic as that observed in either BSC40 cells (Fig. 2-7) or KO3 cells (Fig. 2-8). Nevertheless, the A18R mutant virus is defective in late protein synthesis on DKO cells.

We used northern blot analysis to determine kinetics of mRNA synthesis, and the size and quantity of the steady state mRNAs synthesized in DKO cells. Total cellular RNA extracted from DKO cells infected with wt or C*ts*23 was hybridized with antisense riboprobes specific for early (C11R), intermediate (G8R) or late (F17R) genes (Fig. 3-5). Early C11R mRNAs and intermediate G8R mRNAs in wt and C*ts*23 infected cells are expressed at the same times post infection, in the same quantities, regardless of the incubation temperature. At 31^0C, late F17R mRNA synthesis in C*ts*23 infected KO3 cells is similar to the wt infection at 31^0C. In C*ts*23 infected cells at 40^0C, late F17R mRNA synthesis is initiated at the appropriate time post infection, but the quantities of late mRNA are significantly decreased relative to all other conditions of infection. Consistent with the protein pulse labeling assay, the decrease of late signal is not as dramatic as that in KO3 cell infection (Fig. 2-10). In summary, synthesis of steady state late mRNA in A18R mutant infected DKO cells is reduced in quantity, consistent with the defective late protein synthesis phenotype described above.

In summary, A18R mutant is temperature sensitive in DKO cells and displays a defective late phenotype. This implies that the readthrough transcription affects subsequent gene expression.

Discussion

Previous research shows that the A18R mutant displays a similar phenotype in the RNase L knockout (KO3) cells and in the parental WT1 cells, raising concerns about the true nature of RNase L knockout cell with respect to vaccinia virus infection (Chapter 2). The phenotypic analysis of the growth of an E3L deletion mutant described here has provided a way to differentiate between these two cell lines. The E3L deletion mutant forms plaques in the KO3 cells but not in the WT1 cells, consistent with the lack of RNase L in the knockout cell line. In addition, previous research shows that the A18R mutant is defective in late protein synthesis and mRNA synthesis in KO3 cells (Chapter 2). We predict that the defective late phenotype may result from the activation of the PKR pathway although the RNase L pathway is blocked. Therefore, we investigated the phenotype of an A18R mutant in the RNase L/PKR double knockout cell (DKO). The results show that the A18R mutant also displays a defective late phenotype in the synthesis of protein and steady-state mRNA, which implies that A18R gene is essential in the absence of the RNase L and PKR pathways.

E3L deletion mutant viruses display an intermediate growth phenotype and they form small-sized plaques in KO3 cells. We hypothesize that the low yield of vp1080 in KO3 cells is due to the existence of PKR pathway. Though KO3 cells lack RNase L activity, C*ts*23 infection will produce increased level of dsRNA and will trigger the PKR pathway, which will in turn shut off viral protein synthesis. Future experiments to test this hypothesis will involve comparing the vp1080 growth phenotype in KO3 cells and in

PKR knockout cells which are established from a PKR knockout mouse, and in DKO cells which are established from RNase L/PKR double knockout mouse. We predict that vp1080 will grow well in PKR KO cells because they grow in RK-13 cells which have undetectable PKR activity in the absence of IFN treatment. We predict that vp1080 will also grow well in DKO cells where both RNase L and PKR pathways are absent.

Our phenotypic analysis of the A18R mutant infection in DKO cells provides further insight into the primary consequences of readthrough transcription on the viral infection. Prior phenotypic analysis showed that A18R mutant infections done on KO3 cells displayed a defective late phenotype (Chapter 2). We proposed four possible explanations for this defective late phenotype: (i) the direct involvement of the A18R gene product in late viral transcription initiation; (ii) the inhibition of synthesis of late transcription factors due to the activation of the PKR pathway; (iii) translation interference due to the formation of dsRNA; (iv) transcription interference due to the readthrough transcription. We feel the first possibility is unlikely since *in vitro* experiments from our lab (data not shown) and from other labs (144;314) have failed to demonstrate any role of A18R in initiation of late viral transcription. The second possibility is addressed in this chapter by investigation of the phenotype of A18R mutants in the RNase L/PKR double knockout DKO cells. We have found that in the absence of the activation of both the RNase L and PKR pathway, the A18R mutant virus is still temperature sensitive with respect to virus growth and the steady state late viral mRNAs and late viral proteins are present in reduced amounts. Therefore, the defective late phenotype of A18R mutant on KO3 cells is not mainly caused by the activation of the

PKR pathway. We favor the third and fourth hypotheses: the formation of dsRNA could result in interference with translation of late transcription factors from intermediate mRNAs, also impacting on late mRNA synthesis; and the readthrough transcription could result in direct interference with initiation of transcription from downstream genes. In summary, the A18R gene is essential in the absence of both RNase L and PKR pathway and the readthrough transcription has deleterious effects on the subsequent gene expression.

Fig. 3-1. Two independent pathways signaled by IFN. This figure was taken from Fields' Virology (187). IFN induces the synthesis of the 2-5A synthetase and dsRNA-dependent protein kinase (PKR). Both enzymes are activated by dsRNA produced by viral infection. Activated 2-5A synthetase polymerizes ATP into 2-5A, which in turn activates the constitutively synthesized RNase L. Activated RNase L hydrolyzes mRNA, leading to inhibition of protein synthesis. PKR is activated by dsRNA-mediated autophosphorylation. Activated PKR phosphorylates the α subunit of the translation initiation factor eIF-2. Phosphorylation eIF-2 cannot be recycled from the inactive form to the active form by guanosine exchange factor (GEF). As a result, initiation of protein synthesis is inhibited.

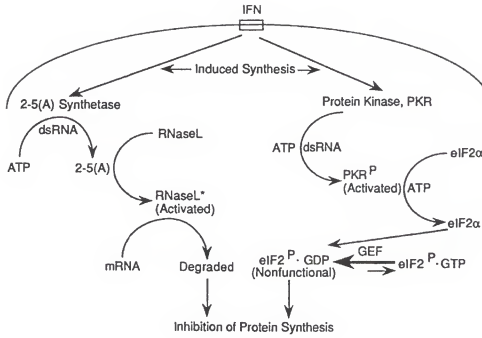

IFN
Induced Synthesis
2-5(A) Synthetase
Protein Kinase, PKR
dsRNA
RNaseL
ATP
2-5(A)
ATP
dsRNA
eIF2α
PKR P
(Activated)
ATP
RNaseL*
(Activated)
eIF2α P
mRNA
Degraded
eIF2 P · GDP
(Nonfunctional)
GEF
eIF2 P · GTP
Inhibition of Protein Synthesis

Fig. 3-2. Plaque assays of vaccinia wt virus and E3L deletion mutant vp1080 on BSC40, WT1 and KO3 cells. Cells were infected with a serial dilution of vp1080 and the infected dishes were incubated at 37^0C for 4 days under liquid media. Media were removed and the cells were stained with crystal violet.

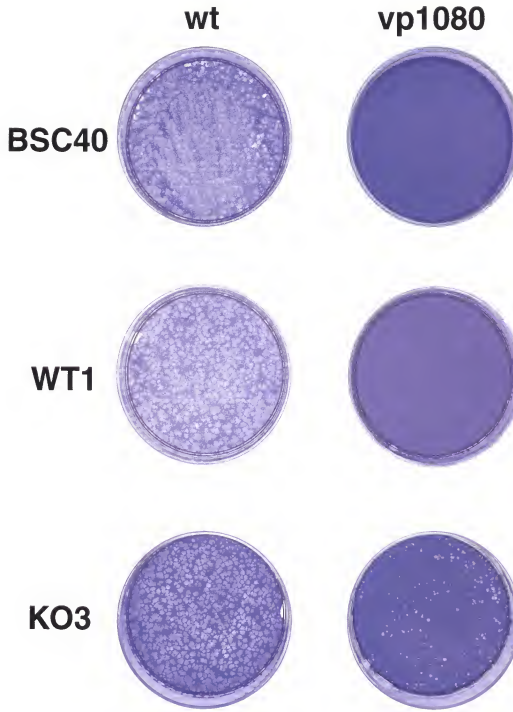

wt
vp1080
BSC40
WT1
KO3

Fig. 3-3. One-step growth of vp1080 in KO3 and WT1 cells. KO3 and WT1 cells were infected at an MOI of 6 with vp1080, and incubated at 37^0C. Samples were taken at various times post infection and virus yields were determined by plaque titration at 37^0C.

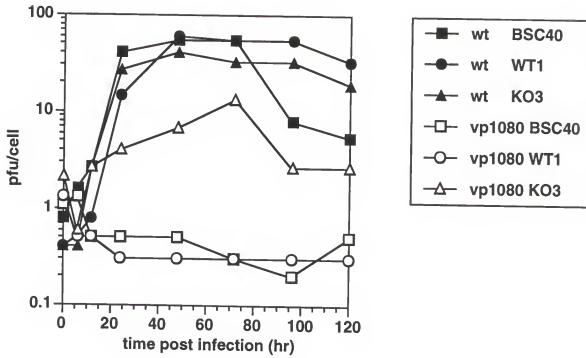

100
10
1
0.1
pfu/cell
0
20
40
60
80
100
120
time post infection (hr)
wt BSC40
wt WT1
wt KO3
vp1080 BSC40
vp1080 WT1
vp1080 KO3

Fig. 3-4. Protein synthesis in wt- and C*ts*23-infected DKO cells. DKO cells were infected with wt or C*ts*23 at an MOI of 15, incubated at 31 or 40^0C, and pulse labeled for 15 min with Trans [^{35}S] Methionine at the times post infection indicated above the lanes in hours. Lanes M, mock infection. Labeled proteins were electrophoresed on SDS-10% polyacrylamide gels and autoradiographed.

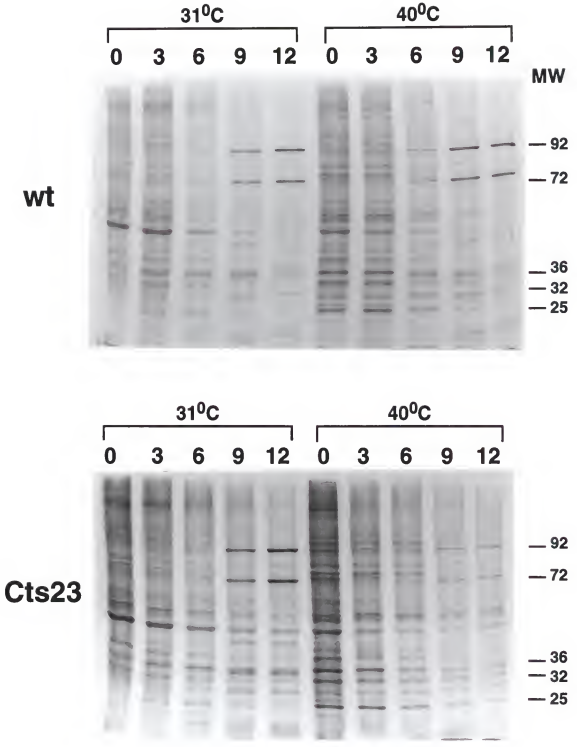
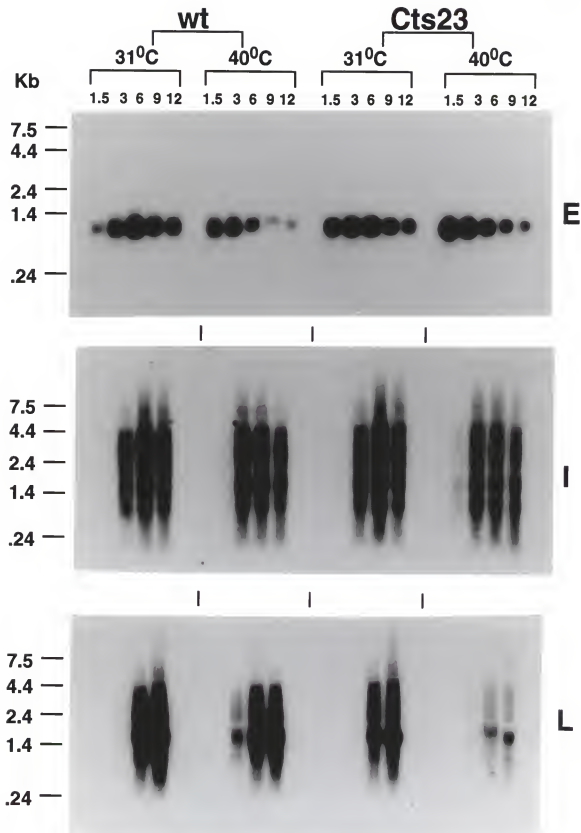

31°C
40°C
0 3 6 9 12 0 3 6 9 12
MW
wt
92
72
36
32
25
31°C
40°C
0 3 6 9 12 0 3 6 9 12
Cts23
92
72
36
32
25

Fig. 3-5. Northern blot analysis of RNA synthesized in wt- and C*ts*23-infected DKO cells. The total RNA was purified from infected DKO cells at various times post infection as indicated on the top of each lane. RNA was analyzed by northern blot analysis using uniformly labeled antisense RNA riboprobes specific for an early (E), intermediate (I), or late (L) gene. Lanes M contain uninfected cell RNA. Sizes are denoted at the left in kb.


wt
Cts23
31°C
40°C
31°C
40°C
Kb
1.5 3 6 9 12 1.5 3 6 9 12 1.5 3 6 9 12 1.5 3 6 9 12
7.5
4.4
2.4
1.4
.24
E
I
L

CHAPTER 4
ISOLATION OF THE EXTRAGENIC SUPPRESSOR OF A18R

Introduction

Late transcription elongation during vaccinia virus infection is subject to both negative and positive regulation, directed by genes A18R and G2R respectively. The vaccinia A18R gene encodes a 56 kD protein that possesses both DNA-dependent ATPase and DNA helicase activities (272). As described in Chapter 2, the A18R phenotype *in vivo* is characterized by readthrough transcription from an upstream intermediate promoter as demonstrated by both RT-PCR analysis in virus infected-BSC40 cells and northern blot analysis in RNase L knockout KO3 cells. Thus A18R encodes a postreplicative negative transcription elongation factor which restricts elongation of viral RNA polymerase during a wt virus infection.

The *in vivo* phenotype of G2R mutant shows that G2R gene plays a role in regulation of late transcription as well. The vaccinia G2R gene encodes a 26 kD protein with unknown function. The predicted amino acid sequence of the G2 protein shows no recognizable functional motifs or homologies to proteins in current databases. The G2R gene is transcribed only early during viral infection (179). G2R mutants isolated in our

lab are the temperature sensitive mutant C*ts*56 and the IBT-dependent deletion mutant G2A. These mutants show a defective late phenotype which is characterized by altered protein synthesis late during infection at the nonpermissive conditions (28). Specifically, protein pulse labeling experiments show that in a G2A infection, late viral protein synthesis begins at the normal time, low-molecular-weight viral proteins are synthesized in normal quantities, but high-molecular-weight viral proteins are synthesized in reduced amounts. RNase protection analysis shows that intermediate and late viral promoters appear to function normally. Northern blot analysis demonstrates that early viral mRNAs are of a proper size and synthesized in normal quantities, but intermediate and late viral mRNAs are reduced in size. Furthermore, RNase protection analysis reveals that these short mRNAs are truncated from the mRNA 3' ends. These data suggest that the G2R protein may regulate the elongation by the viral RNA polymerase late during infection and therefore the G2R protein functions as a positive transcription elongation factor for intermediate and late genes.

Biochemical experiments suggest that the G2R protein directly or indirectly interacts with other viral proteins including A18R and H5R (29). H5R is a 35 kD DNA binding phosphoprotein (212) and it is a substrate for the protein kinase encoded by the virus gene B1R. *In vitro* transcription experiments show that H5R is necessary for transactivation of late genes (144), though the precise role of H5R in stimulating late transcription has not been determined. Affinity purification of a His-tagged G2R protein overexpressed in vaccinia virus-infected cells results in co-purification of the vaccinia virus H5R protein. The interaction between G2R and H5R has also been detected using

the yeast two hybrid system when G2R was cloned downstream of the DNA binding domain and H5R was cloned downstream of the activation domain. Furthermore, immunoprecipitation of infected cell extracts with A18R antibody coprecipitates G2R protein but not H5R protein, while immunoprecipitation with H5R antibody coprecipitates both G2R and A18R proteins. Interestingly, none of these interactions are observed using *in vitro* synthesized His-tagged or GST fusion proteins. Thus, the interactions among A18R, G2R and H5R are not observed in all the assays tested. In summary, H5R probably interacts directly with G2R and it may interact with A18R either directly or indirectly. Similarly, G2R interacts with A18R either directly or indirectly as well as undergoing a direct interaction with H5R.

Treatment of wt vaccinia virus infected cells with the anti-poxviral drug isatin-β-thiosemicarbazone (IBT) results in a readthrough transcription identical to an A18R mutant infection (21;220). This implies that IBT may compromise A18R function directly or indirectly. One IBT resistant mutant of vaccinia has been mapped to the second largest subunit of the viral RNA polymerase, supporting the perspective that the primary target of IBT is the viral transcription apparatus (56). IBT-dependent viruses have been mapped to the G2R gene (179). Specifically, viruses containing deletions in gene G2R grow only in the presence of IBT, and growth of temperature sensitive G2R mutants at the nonpermissive temperature is rescued by IBT. These results suggest that induction of readthrough transcription by IBT can be suppressed by mutation of the G2R gene. Since mutation of the A18R gene also causes readthrough transcription, we hypothesize that mutations in the genes which result in IBT dependence can compensate

for the readthrough transcription caused by an A18R mutant infection. Therefore, we predict that the second site phenotypic revertants of A18R *ts* mutants will map to G2R or some other genes. The experiments described in this chapter were designed to test this hypothesis. The results not only confirm that mutation of G2R suppresses A18R mutations, but also show in addition that another gene, J3R, can serve as an extragenic suppressor of A18R mutations.

Materials and Methods

Cells and Viruses

The continuous African green monkey kidney cell line BSC40 and conditions for cell culture have been previously described (58;59). CV1 cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with nonessential amino acids (Sigma), sodium pyruvate (Sigma) and 10% Fetal Bovine Serum. Wild-type vaccinia virus strain WR, the gene A18R *ts* mutant viruses C*ts*23, C*ts*22, and the gene G2R deletion mutant G2A have been previously described (58;59;179). Briefly, C*ts*22 and C*ts*23 are temperature sensitive mutants of vaccinia virus strain WR that were isolated by random nitrosoguanidine mutagenesis of wt virus. C*ts*22 and C*ts*23 are noncomplementing, they have both been mapped to the A18R gene by marker rescue, and they have identical biochemical phenotypes *in vivo*. The gene G2R mutant G2A is an engineered 10-bp deletion mutant which has its 5' endpoint in codon 90 of the 220-codon gene. The mutation causes a frameshift starting at codon 90 and premature

termination at codon 93 of the mutant sequence. Conditions for virus growth, infection, and plaque assay have been described (58;59). Stock solutions of IBT were made fresh for each use as previously described, and IBT was routinely used at a final concentration of 45 *u*M (221).

DNA Clones

Cosmid clones of wt vaccinia virus (291), the wt HindIII G fragment and the wt HindIII J fragment clones (125) have been described previously. PgG2Rap contains the precise coding region of wt vaccinia virus strain WR gene G2R, cloned in the plasmid vector pGEM3ZF-. It was constructed by PCR amplification of the G2R coding sequence from genomic viral DNA using primers which positioned at the 5' end a synthetic NdeI site, and at the 3' end a synthetic BamHI site. The PCR amplified G2R DNA was trimmed with XbaI and BamHI and cloned into XbaI, BamHI cleaved pGEM3ZF-. The sequence of the insert was confirmed by DNA sequence analysis. PgJ3m clone was constructed by PCR amplification of the J3R coding sequence from genomic r51x4 viral DNA using primers which positioned a synthetic EcoRI site at the 5' end, and a synthetic BamHI site at the 3' end. The PCR amplified J3R DNA was trimmed with EcoRI and BamHI and cloned into EcoRI, BamHI cleaved pGEM3ZF-. The sequence of the insert was confirmed by DNA sequencing analysis. The pJ5 clone was constructed by insertion of the left part of the Hind III J fragment, which was located between the Hind III and Hinc II sites, into the vector pBR322. The pJ6 clone was constructed by insertion of the center part of the Hind III J fragment, which was between

the two Hinc II sites, into the vector pUC9. PgJ3m-7.5gpt clone contains the 7.5k vaccinia promoter sequence driving *E.Coli* guanine phosphoribosyltransferase (gpt) gene, adjacent to the mutant J3R sequence. It was constructed by cloning an EcoRI fragment from pBSgpt4 which contained the 7.5k gpt cassette into the EcoRI cleaved pgJ3m. PET14-J3R which contains the precise coding region of the wt vaccinia virus gene J3R, was kindly provided by Dr. Edward Niles (SUNY Buffalo).

Marker Rescue

Marker rescue was done as previously described (291), except that DNA was transfected into infected cells using lipofectin (Life Technologies, Gaithersberg, MD) following the manufacture 's instructions.

DNA Sequencing

DNA sequence of A18R, G2R and J3R mutations was obtained by direct sequencing of PCR products amplified from genomic viral DNA. Genomic viral DNA was obtained by any of the three methods: (1) Large-scale preparation of viral DNA were purified from viral cores isolated from cytoplasmic extracts of infected cells as previously described (59). (2) Small-scale preparations of total cytoplasmic nucleic acid containing viral DNA were made using a modification of the large-scale procedure as follows (60). Briefly, confluent 60-mm dish of BSC40 cells was infected with virus at a MOI of 1 and incubated under appropriate conditions until a complete cytopathic effect was observed. Cells were harvested, washed with isotonic buffer, resuspended in 250 ul of hypotonic

buffer, and frozen and thawed once. Cells were lysed by addition of 25 *u*l of 10% Triton X-100 and 1 ul β-mercaptoethanol and incubation on ice for 60 min. Nuclei were removed by low-speed centrifugation, and the cytoplasmic supernatant was removed, adjusted to 1% SDS, 100 *u*g/ml proteinase K, 65 mM NaCl, and incubated at 45^0C for 6 hr. Nucleic acids were then purified from the cytoplasmic extract by phenol extraction and ethanol precipitation. The final pellet was resuspended in 50 *u*l TE, and 1 *u*l was used in PCR reactions. (3) Infected cell lysates prepared by standard methods (58;59) were used directly for PCR. Lysates contain 1x 10^7 infected cells per milliliter in PBS and have been frozen and thawed at least twice. Five microliters of lysate was added to 95 *u*l of TE, boiled for 5 min, quick cooled, and 70 *u*l of this preparation was used as a source of template in a 100 *u*l PCR reaction.

For DNA sequence analysis, the 1482-nt A18R coding sequence was amplified using five different primer pairs which produced overlapping double-stranded PCR products ranging in size from 394 to 533 bp. The 663-nt G2R coding sequence was amplified using three different primer pairs which produced overlapping double stranded PCR products ranging in size from 337 to 389 bp. The 1189-nt J3R coding sequence was amplified using two different primer pairs which produced overlapping double stranded PCR products ranging in size from 635 to 636. In the case of G2R and A18R, the upstream primers contained at the 5' end the 18-nt M13 reverse universal primer sequence and was 5' biotinylated. DNA products from 100-*u*l PCR reactions were purified using Wizard PCR preps (Promega Corp., Madison WI) columns and eluted in a final volume of 50 *u*l water. Strand separation and DNA sequence analysis were done by

the University of Florida BEECS Genetic Analysis Core and the ICBR DNA Sequencing Core Laboratory. Briefly, the PCR products were mixed with streptavidin-conjugated paramagnetic beads. The beads were retained with a magnet and then the nonbiotinylated strand was released by treatment with alkali while biotinylated DNA was retained on the beads. This procedure yields alkali-released single-stranded template DNA which has at its 3' end the complement of the M13 reverse universal primer sequence. DNA was sequenced using dye-labeled primers and an Applied Biosystems Model 373A DNA sequencer. Mutations were confirmed and ambiguities were resolved by re-sequencing the same strand or by sequencing isolated portions of the complementary strand using a double-stranded PCR product as DNA template, appropriate DNA sequencing primers, and dye-terminated DNA sequencing reactions. In the case of J3R, the upstream primers did not contain universal primer sequence and were not biotinylated. The DNA products from PCR reactions were purified using Wizard PCR preps and both strands of DNA were sequenced directly by the University of Florida BEECS Genetic Analysis Core and the ICBR DNA sequencing core laboratory.

Transient Dominant Selection

Transient dominant selection was performed as previously described (115). Briefly, CV1 cells were pretreated with mycophenolic acid (MPA), infected with wt vaccinia virus at MOI of 0.05 pfu/cell, transfected with 20 ug of plasmid DNA using Transfectase (Gibco-BRL), and incubated at 37^0C for 3 days. Infected, transfected cells were harvested and titrated for plaque formation on CV1 cells in the presence of MPA.

Individual MPA-resistant plaques were picked and titrated for plaque formation on BSC40 cells in the presence and absence of IBT. Individual IBT-dependent plaques were picked and replaqued in the presence and absence of IBT. The J3R gene from mutant viral DNA was amplified by PCR and subsequently sequenced.

Results

Mutant Isolation

As an initial test of the hypothesis that lethal mutation of both A18R and G2R genes would yield a viable virus, a recombinant between an A18R *ts* mutant (C*ts*23) and an IBT-dependent G2R deletion mutant (G2A) was constructed. BSC40 cells were infected with a mixture of C*ts*23 and G2A at a MOI of 5 for each virus. Infections were incubated at 31^0C for 72 hr in drug-free medium and then titered at 31^0C and 40^0C in the absence or in the presence of IBT. Most of the viruses which form plaques at 40^0C in the absence of IBT are recombinants. Five plaques were picked and retested by plaque assay at 31^0C or 40^0C in the absence or in the presence of IBT. Of these five isolates, two appeared wt in plaque phenotype, one appeared to contain a mixture of C*ts*23 and G2A viruses, and two formed smaller than wt plaques at both temperatures in the absence of IBT. One of the latter two isolates, named x41, was grown and used for further study.

Next, we attempted to isolate extragenic suppressors of another gene A18R *ts* mutant, *Cts*22. Reversion was induced by chemical mutagenesis (58). BSC40 cells were infected with *Cts*22 at an MOI of 10 and incubated at 31^0C in growth medium containing

10 *u*g/ml nitrosoguanidine. At 10 hr post infection, the medium was replaced with fresh growth medium lacking mutagen. The virus was harvested at 48 hr post infection. Mutagenized virus was diluted to a concentration of 1 x 10^6 PFU/ml, seeded on 25 100-mm dishes at an MOI of 0.16, overlayed with nutrient agar, incubated at 40^0C for 4 days, and stained overnight with neutral red. On Day 5, a total of 12 plaques were observed, all of which were isolated and tested directly for plaque formation at 31 and 40^0C. Of these 12 plaques, two cold sensitive mutants (named cs1 and cs4) were isolated.

We also attempted isolation of spontaneous extragenic suppressors of C*ts*23. A stock of C*ts*23 containing approximately 10^{-3} revertants was plated at an appropriate dilution at 40^0C, and 20 plaques growing at 40^0C were isolated and tested for plaque formation at 31 and 40^0C + and - IBT. Two of these isolates formed smaller than wt plaques at both temperatures. These two isolates, named r41 and r51, were grown and used for further study. No cs isolates were detected in this experiment.

In summary, we have isolated five new putative double mutant viruses for further study. These consists of a C*ts*23 and G2A recombinant named x41, two induced cs revertants of C*ts*22 named cs1 and cs4, and two spontaneous small-plaque, ts^+ revertant of C*ts*23, named r41 and r51. The plaque phenotype of all these viruses relative to parental viruses is shown in Fig. 4-1. The mutants were evaluated further by test cross analysis with wt virus (data not shown), marker rescue (Fig. 4-2 and 4-3), and by DNA sequence analysis (Table 4-1), as described below.

Mutation of G2R Gene Causes Suppression of A18R Gene *ts* Mutants

We mapped the mutations causing cold sensitivity in cs1 and cs4 by marker rescue. BSC40 cells were infected at low MOI with either cs1 or cs4 and transfected with various DNA fragments cloned from wt virus. Infected, transfected cells were incubated under conditions which are nonpermissive for the cs mutants, 31^0C -IBT, for 6 days, and stained with crystal violet. Successful rescue of the cs marker is indicated by the appearance of plaques on the stained dishes. Rescue was tested with cosmid clones which span the entire vaccinia genome (not shown), with the 8.9-kb G2R gene-containing HindIII G fragment (not shown), and with pgG2Rap, a clone which contains only the G2R coding sequence (Fig. 4-2). The mutant cs1 was rescued well with two overlapping cosmid clones (pWR 45-83 and pWR 67-98), both of which contain the G2R gene. The mutant cs1 was not rescued with any of the other cosmid clones tested, none of which contain the G2R gene. The mutant cs1 was also rescued with HindIII G, and most importantly, with pgG2Rap (Fig. 4-2). Rescue of cs4 was not as efficient as that of cs1, and therefore convincing rescue was not observed with pWR45-83, although positive rescue was observed with pWR 67-98 and with HindIII G (not shown). Positive marker rescue of cs4 was also observed with pgG2Rap (Fig. 4-2). These results prove that the mutation which causes cold sensitivity in cs1 and cs4 maps to gene G2R.

To determine the genotype of the mutant viruses, DNA sequence analysis was performed on PCR products from genomic mutant viral DNA corresponding to genes A18R and G2R. In cases where the sequence of a previously unknown mutation in a gene was being determined, the entire gene in question was sequenced. In cases where

the analysis was done in order to confirm the presence of a known mutation, only the relevant region of DNA was sequenced. The results of the sequence analysis are summarized in Table 4-1. Analysis of the entire A18R gene from C*ts*22 and C*ts*23 revealed a single, unique, point missense mutation in each case. C*ts*22 contains a C to T transition in codon 46 of gene A18R, resulting in substitution of serine for proline. C*ts*23 contains a C to T transition at codon 432 of gene A18R, resulting in substitution of phenylalanine for serine. The G2A deletion mutant contains a frameshift and premature translation termination. DNA sequence analysis of the C*ts*23-G2A recombinant, x41, confirmed that it contained both the G2A and C*ts*23 alleles. The ts^+ revertant of Cts23, r41, contained the C*ts*23 allele of gene A18R and in addition contained a missense mutation in codon 102 of gene G2R, resulting in substitution of aspartic acid for glycine. The cs revertants of C*ts*22, cs1 and cs4, each contained the C*ts*22 allele and in addition each contained a missense mutation in codon 102 of the G2R gene. Mutation in cs1 results in substitution of serine for glycine, while mutation in cs4 results in substitution of aspartic acid for glycine, which is identical to the mutation found in r41. We conclude from the DNA sequence analysis that the recombinant virus x41 contains both parental mutant alleles and that the revertants r41, cs1, and cs4 each contain the parental A18R mutant allele plus a mutation in codon 102 of gene G2R.

Mutation of J3R Gene Causes Suppression of A18R Gene *ts* Mutants

One revertant of C*ts*23, named r51, contains the parental A18R mutant allele and a wildtype G2R gene. In order to identify the other mutant allele of this virus, r51 was

test crossed with the wildtype virus to segregate the A18R mutation from the suppressing mutation. The cross yielded an IBT^d virus (r51x4) in addition to parental viruses. This result indicated that the suppressing allele was phenotypically IBT^d when expressed in isolation from the A18R mutation. We then mapped the mutation causing IBT dependence in r51x4 by marker rescue. BSC40 cells were infected at low MOI with r51x4 and transfected with various DNA fragments cloned from wt virus. Infected, transfected cells were incubated under conditions which are nonpermissive for the r51x4, -IBT at 37^0C, for 6 days, and then stained with crystal violet. Successful rescue of the IBT^d marker is indicated by the appearance of plaques on the stained dishes. Rescue was tested with cosmid clones which span the entire vaccinia genome (not shown), and with the plasmid containing HindIII J fragment (not shown), and with PET14-J3R, a clone which contains only the J3R coding sequence (Fig. 4-3). The mutant r51x4 was rescued well with three overlapping cosmid clones (pWR 45-83, pWR 67-98 and pWR 74-111), each of which contain the J3R gene. The mutant r51x4 was not rescued with any of the other cosmid clones tested, none of which contain the J3R gene. The mutant R51x4 was also rescued with HindIII J, and most importantly, with PET14-J3R (Fig. 4-3).

To determine the genotype of the mutant viruses, DNA sequence analysis was performed on PCR products from genomic mutant viral DNA corresponding to genes A18R and J3R. This analysis reveals that the r51x4 allele contains two missense mutations. One mutation is a G to A transition in codon 96 of gene J3R, resulting in substitution of aspartic acid for glycine (G96D mutation). The other mutation affects the region where the C-terminus of the J3R and the N terminus of the J4R gene overlap out

of frame. The mutation results in substitution of lysine for arginine at codon 327 of gene J3R (R327K mutation) and asparagine for aspartic acid at codon 22 of J4R (Fig. 4-3).

In order to determine which one of the two mutations is responsible for suppression of A18R, we used marker rescue analysis with two plasmids. Plasmid pJ5 contains the 5' end of the J3R coding sequence, while plasmid pJ6 contains the overlap sequence of the 3' end of the J3R and the 5' end of J4R. BSC40 cells were infected at low MOI with r51x4 and transfected with either the pJ5 clone or the pJ6 clone. Infected, transfected cells were incubated under conditions which are nonpermissive for r51x4, - IBT 37^0C, for 6 days, and stained with crystal violet. The mutant r51x4 was only rescued with the pJ5 clone but not with the pJ6 clone, suggesting that the G96D mutation causes IBT dependence (Jeremy Condit unpublished). We conclude from the marker rescue and DNA sequencing analysis that the revertant virus r51 contains the G96D mutation in addition to the parental A18R mutant allele, and that the G96D mutation causes IBT dependence of virus r51x4. This result implies that mutation of J3R gene causes suppression of A18R *ts* mutation.

In order to prove conclusively that the J3R mutation suppresses A18R *ts* mutation, we attempted to construct a virus containing only the J3R G96D mutation and to recombine that allele into A18R *ts* mutant to test whether the resulting recombinant is phenotypically wild type. We used two strategies to construct the J3R single mutant which contains only the G96D mutation. First, we used transient dominant selection by transfecting wt-infected CV1 cells with the pgJ3m-7.5gpt clone and selecting mycophenolic acid resistant recombinants which should contain a mutant copy of J3R.

Individual mycophenolic acid resistant recombinants were picked, titrated for plaque formation and further plaque purified. The virus selected and purified using the transient dominant selection was named J3T. Second, test crossing of r51x4 with wt virus yielded an IBT^d virus in addition to the parental viruses. The virus selected and purified from the test cross was named J3X. Since both the G96D mutation and the R327K mutation destroy the restriction enzyme sites in the wt sequence, the new viruses containing only the G96D mutation were distinguished from wt and r51x4 viruses by restriction enzyme digestion. We purified genomic mutant viral DNA from both J3X and J3T. We then verified the sequence by restriction digestion analysis and DNA sequencing analysis. This J3R mutant virus, named J3X, is further characterized in Chapter 5. To recombine the J3R mutant allele back to the A18R mutant virus, we constructed a double mutant of A18R and J3R. We have isolated phenotypically wt virus and the genotype of this recombinant needs to be verified by DNA sequencing analysis.

In summary, G2R and J3R act as second site suppressors for the A18R mutation. We interpret these results to mean that A18R and G2R, A18R and J3R interact with each other either directly or indirectly in a fashion which affects intermediate and late viral transcription.

Discussion

Our results, summarized in Table 4-1, demonstrate that mutation of both the vaccinia virus G2R and J3R genes can compensate for mutations in gene A18R.

Previous experiments with IBT suggest a relationship between the A18R and G2R genes. Treatment of wt virus infected cells with IBT results in a similar readthrough phenotype as A18R mutant infections (21). Lethal mutation of the G2R gene results in IBT dependence (179) and shorter transcripts (28). It suggests that lethal mutation of the G2R gene can offset the readthrough effects of transcription caused by IBT treatment or A18R mutation. Therefore, A18R and G2R could play counteracting roles in regulating transcription elongation. The results I have presented in this chapter support this hypothesis. First, a recombinant virus x41, which contain both an A18R *ts* mutation and a G2R deletion mutation, is viable at 40^0C. Second, genetic data demonstrate that the extragenic suppressors of A18R *ts* mutations (r41, cs1 and cs4) all affect the same G2R gene.

The genetic evidence for interaction between A18R and G2R is consistent with the *in vivo* phenotypic analysis and *in vitro* biochemical analysis. The *in vivo* phenotypic analysis suggests that A18R is a postreplicative termination factor, and G2R is a postreplicative positive transcription elongation factor. In the absence of A18R function, long transcripts are formed late during viral infection (Chapter 2). In the absence of G2R, short transcripts are formed late during viral infection (28). In the cases where both A18R and G2R are missing, balance would be restored to transcription elongation and properly sized transcripts were produced. In addition, the previous biochemical analysis shows that G2R interacts with A18R either directly or indirectly and interacts directly with a late transcription factor, H5R, whereas H5R interacts with A18R either directly or indirectly (29).

Two additional interesting observations were obtained from the genetic assay. First, the three independently isolated extragenic suppressors of A18R *ts* mutations affect the G2R gene at the same codon, Codon 102. This implies that that codon 102 is extremely critical for G2R function. It is interesting that similar or identical G2R mutations result in two different phenotypes when combined with two different A18R *ts* alleles. When G2R mutations are combined with C*ts*23, the phenotype is *ts*+, whereas when similar or identical mutations are combined with C*ts*22, the phenotype is cs. This observation implies that the two A18R *ts* alleles are functionally different. Second, plaque phenotype analysis showed that all of the double mutants are IBT^s. However, since both IBT treatment and A18R mutations cause promiscuous transcription, we predicted that any second site mutation that compensated for an A18R mutation would also be compensated by A18R for the effects of IBT. In this case, the A18R-G2R double mutants would be insensitive to IBT. This contradiction implies that IBT may target a gene other than A18R or G2R. We contend that the most likely candidate for the primary target of IBT is the viral RNA polymerase, since one IBT^r mutant has been mapped to the second largest subunit of RNA polymerase (56).

R51, a spontaneous revertant of C*ts*23, contains a mutation in gene A18R, but a wt G2R allele (Table 4-1). Test cross of r51 with wt virus produces the progeny virus r51 x 4 which is IBT^d. Further marker rescue and DNA sequencing analysis show that the J3R G96D mutation causes IBT dependence and suppresses A18R mutation. This novel finding implies that J3R is probably involved in the transcription regulatory complex and inspired us to investigate the phenotype of r51 x 4. Similar to the G2R deletion mutant,

r51 x 4 displays a defective late phenotype (Latner unpublished). Specifically, under nonpermissive conditions (–IBT), synthesis of late viral proteins with high-molecular-weight is reduced in amount while synthesis of late viral proteins with low-molecular-weight is of normal amounts. In addition, northern blot indicates that early mRNAs have similar sizes as in a wt infection, while intermediate and late mRNAs are reduced in size. Whether these mRNAs are truncated from the mRNA 3' ends and whether promoter utilization is normal remains to be determined. However, these preliminary *in vivo* data imply that the J3R protein may function to regulate transcription elongation late during infection.

Based on the analysis of the G2R and J3R mutations described here, we predict that mutations which result in IBT dependence affect genes which can compensate for the readthrough transcription caused by an A18R mutant infection. Don Latner therefore isolated several independent IBT^d viruses in order to identify other genes which might serve as extragenic suppressors of A18R. Interestingly, among the nine isolates, two have been mapped to the G2R gene, and seven have been mapped to the J3R open reading frame (Latner unpublished). It is not yet known whether these IBT-dependent viruses will suppress an A18R *ts* mutant. One of the J3R mutants, named J3D, contains a T deletion at position 142 resulting in a frameshift and introduction of a premature stop codon. The resulting protein is predicted to be about 58 amino acids long (6.4kD). This virus is further characterized for polyadenylation function *in vivo* in Chapter 5.

In prokaryotic and eukaryotic systems, transcription elongation is influenced by cis-acting sequences, trans-acting factors and the RNA polymerase. Positive and

negative transcription factors are balanced to regulate transcription elongation. In the absence of positive elongation factors, the RNA polymerase will produce aborted transcripts because of the function of negative transcription elongation factors. In the presence of positive elongation factors, RNA polymerase will produce full-length transcripts (169). The results reported here combined with genetic, biochemical and *in vivo* phenotype analysis data, suggest that similar to prokaryotic and eukaryotic transcription factors, A18R does not act alone but rather as a part of a larger complex that serves to regulate postreplicative gene elongation and termination. The function of G2R protein may be to promote transcription through pause sites as a positive transcription elongation factor. The ATPase activity of A18R may promote the termination of the transcription and release of the transcripts and RNA polymerase. IBT may target the viral RNA polymerase directly because the IBT resistance maps to an RNA polymerase subunit. How J3R protein fits in this large complex and which function of the J3R protein, the methyltransferase activity or the polyadenylation stimulatory function, is important for suppression will be addressed in Chapter 5. It is likely that other protein factors are involved in the late transcription complex as well. We expect to continue the genetic strategy to uncover additional genes which might influence vaccinia transcription in collaboration with A18R, G2R and J3R.

Fig. 4-1. Plaque phenotypes of A18R and G2R mutant viruses. Confluent monolayers of BSC40 cells were infected with 50-100 PFU per dish of wt or mutant virus. Infected dishes were incubated at 31^0C (31) or 40^0C (40) in the presence (+) or absence (-) of IBT for 1 week under a nutrient agar overlay. Overlays were removed and the cells were stained with crystal violet.

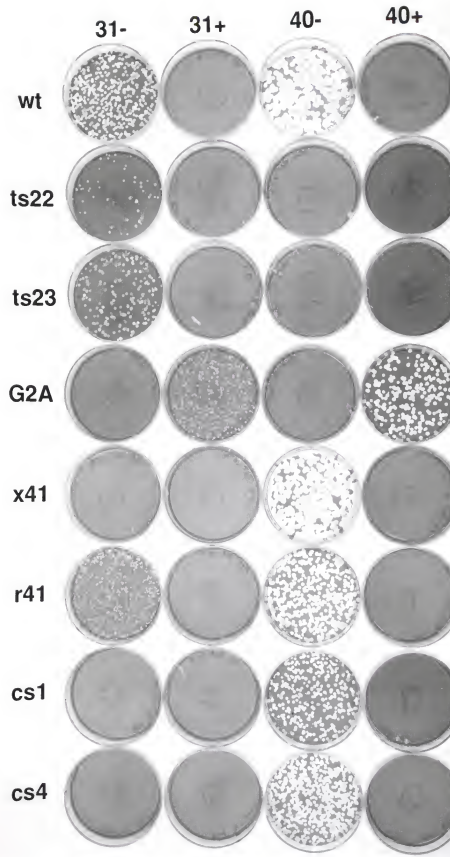

31-
31+
40-
40+
wt
ts22
ts23
G2A
x41
r41
cs1
cs4

Fig. 4-2. Marker rescue of cs1 and cs4. Confluent monolayers of BSC40 cells were infected at low MOI with either cs1 or cs4 and transfected with pgG2Rap, which contains the G2R gene (+G2R), or mock transfected (-DNA). Infected, transfected cells were incubated at 31^0C -IBT for 6 days and then stained with crystal violet. Successful rescue of the cs marker is indicated by the appearance of plaques on the stained dishes.

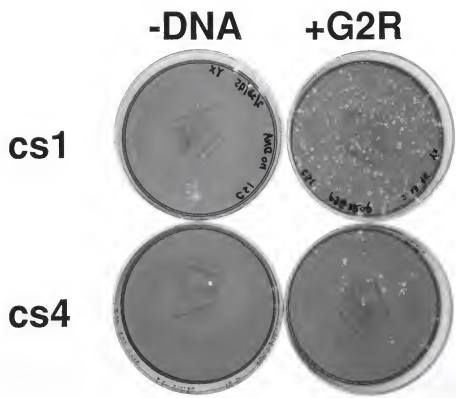
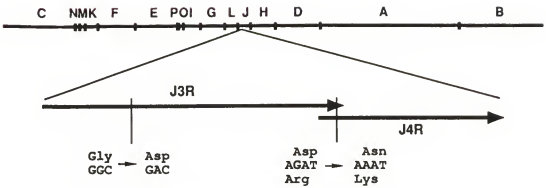
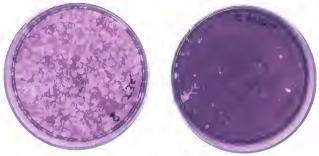

-DNA
+G2R
cs1
cs4

Fig. 4-3. Marker rescue of r51x4. Confluent monolayers of BSC40 cells were infected at low MOI with r51x4 and transfected with a clone PET14-J3R containing the J3R ORF (+J3R) or mock transfected (-DNA). Infected, transfected cells were incubated at 37^0C - IBT for 6 days and then stained with crystal violet. The J3R and J4R genes are drawn to scale and they overlap each other out of frame. The mutation at codon 96 affects only the J3R gene and results in Gly to Asp change. The mutation at codon 327 of J3R and codon 22 of J4R results in Asp to Asn change in J3R and Arg to Lys change in J4R.


C
NMK
F
E
POI
G
L
J
H
D
A
B
J3R
J4R
Gly
GGC
Asp
GAC
Asp
AGAT
Arg
Asn
AAAT
Lys



J3R - DNA

Table 4-1
A18R Extragenic Suppressors

Virus	A18[a]	J3[a]	G2[a]	Phenotype[b]
wt	+	+	+	ts+, IBT^s
ts22	P46S	+	+	ts+, IBT^s
ts23	S432F	+	+	ts+, IBT^s
G2A	+	+	Δ90–220	ts+, IBT^d
x41[c]	S432F	+	Δ90–220	ts+, IBT^s
r41[d]	S432F	+	G102D	ts+, IBT^s
r51[d]	S432F	G96D	+	ts+, IBT^s
cs1[e]	P46S	+	G102S	cs, IBT^s
cs4[e]	P46S	+	G102D	cs, IBT^s

a Describes the genotype of each virus with respect to either the A18, J3, or G2 gene. + = wild type; P46S = substitution of proline for serine at codon 46; Δ90-220 = deletion of codons 90-220.

b A descriptive summary of the plaque formation phenotype

c A recombinant between ts23 and G2A

d A second site revertant of ts23

CHAPTER 5
BIOCHEMICAL ANALYSIS OF AN EXTRAGENIC SUPPRESSOR OF A18R - J3R

Introduction

Historically vaccinia virus has served as an excellent model for the study not only of transcription but also of 5' and 3' end modification of mRNA. First, the early viral RNAs were shown to synthesize 100 to 200 adenylates at their 3' ends using vaccinia virus cores in the presence of four ribonucleoside triphosphate (133;134). Later, viral RNAs were found to be blocked at their 5' ends by two adjacent methylated nucleotides in a triphosphate linkage when purified vaccinia virus particles were incubated with S-adenosylmethionine (307). These data suggest that enzymes present within the virus cores are capable of synthesizing mRNA with eukaryotic characteristics including a poly (A) tail at the 3' end and a cap structure at the 5' end. Disruption of the virus core with detergent results in release of several enzymes including a poly (A) polymerase (PAP) and enzymes involved in cap formation: an mRNA guanylyltransferase, an mRNA (guanine-7-)-methyltransferase, and an mRNA-(nucleoside-2'-O-)-methyltransferase.

As described in Chapter 1, the vaccinia poly (A) polymerase has been purified from virus cores or virus-infected cells as a heterodimer, containing the VP55 and VP39

subunits (205;206). The genes encoding these two polypeptides were later found to be E1L and J3R, respectively (94). It is estimated that about 110 molecules of poly (A) polymerase are present in each virion. In virion extracts, VP55 is present only in association with VP39, while VP39 is present in both heterodimeric and monomeric forms. The VP55-VP39 heterodimer catalyzes the formation of poly (A) tails several hundred nucleotides in length *in vitro*. The activity of this enzyme has an optimum pH of about 8.6, and is dependent on divalent cations. It adds polyadenylates with no primer sequence specificity *in vitro* (191). The heterodimer could be dissociated by incubation with antibody specific for N-terminal of VP55 and the resulting VP55 protein has been found to possess the catalytic polyadenylation function. There is no polyadenylation catalytic function associated with VP39 (94). Instead, VP 39 can specifically bind to free poly (A) using a poly (A) binding assay and further chromatography (97). When VP55 and VP39 are overexpressed individually, VP55 by itself acts to add 30-35 adenylates to an RNA primer in a rapid and processive burst and then leads to a nonprocessive polyadenylation *in vitro* (94). Addition of VP39 to the VP 55 catalyzed polyadenylation reaction stimulates the formation of long poly (A) tail by converting the slow polyadenylation to a rapid reaction (97).

The mRNA capping activities were purified from vaccinia virions at the same time as the vaccinia poly (A) polymerase was purified (171;173). Guanylyltransferase and guanine-7-methyltransferase activities have been isolated as a 127 kD complex consisting of two polypeptides that are responsible for forming a cap 0 structure on mRNA (171). The genes coding for this protein complex, D1L and D12L, have been

identified (184;207). An mRNA (nucleoside-2'-O-)-methyltransferase has also been purified from vaccinia virus by column chromatography (19). This isolated 39 kD enzyme has an optimum pH of 7.5 and does not require divalent cations. It has a Km of 2.0 uM for S-adenosylmethionine and a Km of 5 mM for brome mosaic virus RNA (18). The gene encoded for this 39 kD protein has been identified to be J3R (253), the same gene that encodes the small subunit of poly (A) polymerase. It has been shown that the methyltransferase activity is associated with both the monomeric VP39 protein and the heterodimeric VP55-VP39 complex (255).

In summary, vaccinia virus J3R gene encodes a VP39 protein that has both mRNA 5' cap-specific (nucleoside-2'-O-)-methyltransferase and 3' polyadenylation stimulatory activities. At the mRNA 5' end, VP39 converts the cap 0 structure (m^7G(5')pppG/A) to the cap 1 structure (m^7G(5')pppG^m/A^m) by methylating the ribose 2'-OH of the first transcribed nucleotide in a S-adenosylmethionine (AdoMet)-dependent manner. At the 3' end of mRNA, VP39 stimulates poly (A) tail elongation as the smaller subunit of the heterodimeric vaccinia virus poly (A) polymerase (94). VP39 exists both in monomeric form and in association with VP55 in the virion. Schnierle et al used both deletion and alanine scanning mutagenesis to identify which regions of VP39 mediate its two functions (254). They found that deletion of C-terminal 26 amino acids had no detectable effect on either activity *in vitro*, while deletion of C-terminal 75 or 112 amino acids resulted in the loss of both activities. The deletion of C-terminal 75 amino acids decreased RNA binding, while deletion of 112 amino acids abolished RNA binding. Eleven of 21 charge cluster to alanine mutations had very low methyltransferase activity

in vitro. Among the 11, four mutant proteins also lacked the polyadenylation stimulation activity. No mutant protein has been isolated so far that only affects the polyadenylation but not the methylation activity. Recently, an X-ray crystal structure of the AS11 mutant of VP39 complexed with its AdoMet cofactor has been reported (261). Since wt VP39 could not be crystallized, they used AS11 mutant which contains R140A, K142A and R143A, and which retains polyadenylation processivity activity. The structure shows that VP39 comprises a single core domain with structural similarity to the catalytic domains of other methyltransferases. The catalytic domain and associated binding sites for VP39's methyltransferase function is in close proximity to a superficial cleft on the protein surface.

We have provided genetic evidence that mutation of J3R serves as a second site suppressor of an A18R mutation, indicating that these two proteins probably interact with each other or function in the same pathway to regulate viral elongation and/or termination (Chapter 4). The experiments described here were designed to understand the significance of polyadenylation processivity and/or the methyltransferase activity of J3R in suppression of the A18R mutation. It will help us to understand better the function of the A18R protein in transcription and mRNA metabolism. Polyhistidine tagged J3R wt and mutant proteins were purified from bacterial cells and analyzed for biochemical activities in methyltransferase activity assays and adenylyltransferase stimulatory function assays. The results show that the ribose-2'-O-methyltransferase activity is defective with the J3R mutant protein while the polyadenylation function is normal

compared to a wt J3R protein. The length of the poly (A) tail of the J3R single mutant virus *in vivo* is normal compared to that of the wt virus.

Materials and Methods

Cells and Viruses

The continuous African green monkey kidney cell line BSC40 and conditions for cell culture have been previously described (58;59). Wild-type vaccinia virus strain WR, and the IBT^d J3R mutants, the conditions for their growth, infection, and plaque titration have been described previously (58;59). As described in Chapter 4, r51x4 contains two missense mutations, one in condon 96 of gene J3R, resulting in substitution of aspartic acid for glycine (G96D mutation), and the other affecting the region where the C-terminal of J3R (at codon 327) and the N terminal of J4R gene (at codon 22) overlapping out of frame. J3X contains only the G96D mutation. J3D contains a deletion at codon 142, resulting in a frameshift and premature termination. The resulting protein is predicted to be 58 amino acids in length.

Plasmid Constructs

PET32a-J3RG96D contains the precise coding region of the vaccinia virus J3R gene with the G96D mutation cloned in the expression vector pET32a (Novagen). The N-terminus of the vector contains thioredoxin-tag and polyhistidine-tag sequences. PET32a-J3RG96D was constructed by PCR amplification of the J3R coding sequence

from genomic r51x4 vial DNA using primers which positioned a synthetic NcoI site at the 5' end, and a synthetic EcoRI site at the 3' end. The PCR amplified J3R DNA was trimmed with NcoI and EcoRI and cloned into NcoI and EcoRI cleaved pET32a. The 3' primer contains a mismatch such that by PCR amplification it corrects the mutation at codon 327 of gene J3R in r51x4 back to wt sequence. The sequence of the insert was confirmed by DNA sequence analysis.

PET32a-J3RG96D was transformed into bacterial strain AD494(DE3)pLysS according to the pET system manual (Novagen). Briefly, 5 ng of DNA was mixed with 20 μl of competent AD494(DE3)pLysS cells, the mixture was placed on ice for 30 min, heated at 42^0C for 40 seconds, and then placed on ice for 2 min. Then 80 μl of LB solution was added to the mixture. After shaking at 200 rpm at 37^0C for 1 hr, 50 μl of each transformation was spread on the LB plate with appropriate antibiotics. The plates were incubated at 37^0C overnight. A single colony was picked the next day for protein expression and purification.

Bacterial strains AD494(DE3)pLysS-pET32aE1L and AD494(DE3)pLysS-pET32aJ3R were kindly provided by Dr. Edward Niles (SUNY, Buffalo). AD494(DE3)pLysS-pET32aE1L is a bacterial strain expressing wt E1L protein and AD494(DE3)pLysS-pET32aJ3R is a bacterial strain expressing wt J3R protein.

Purification of His-tagged J3R and E1L Proteins

The bacterial overexpression of pET32a-G96D and purification of the His-tagged proteins were undertaken according to the pET system manual (Novagen). Briefly,

bacteria were grown in 50 ml LB containing 150 ug/ml ampicillin and 34 ug/ml chloramphenicol at 37^0C overnight. Thirty milliter of the preculture was used to inoculate a three-liter culture. After the OD_{600} of the three-liter culture reached 0.6, the culture was induced by addition of IPTG (100 mM stock) to a final concentration of 0.1 mM and further incubated at room temperature overnight. Bacterial cells were sedimented, the pellets were resuspended in 40 ml of lysis buffer [1 x binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris HCl, pH 7.9), 1 mM PMSF, 0.01% NP40, 1 mM $MgCl_2$ and 2.5 mg DNase I] and placed on ice. Extracts were made by sonication and then supernatant was collected and applied on a precharged Ni^{++} column. The column was washed with 10 Volumes of binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris HCl, pH 7.9) and then with 6 volumes of washing buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris HCl, pH 7.9). The proteins were eluted from the column with 6 volumes of elution buffer (100 mM imidazole, 500 mM NaCl, 20 mM Tris HCl, pH 7.9). Fractions containing proteins were pooled and dialyzed in Buffer A (25 mM Tris HCl, pH 8, 1 mM EDTA, 10 mM β-mercaptoethanol, 50 mM NaCl and 10% glycerol). The pooled dialyzed J3R proteins were then loaded on a 2.5 ml Q-sepharose column, and eluted using 2 x 20 ml elution buffer containing a gradient of 50 mM to 500 mM NaCl in Buffer A. Protein concentration was determined by the Bradford assay, using bovine serum albumin (BSA) as the standard. Equal volumes of purified proteins were fractionated on SDS-PAGE using 10% separating gels. The gels were stained with Coomassie blue for 40 minutes and then destained overnight in the buffer containing 20% methanol and 7% glacial acetic acid.

Preparation of Brome Mosaic Virus RNA (BMV RNA)

Purified brome mosaic virus was kindly provided by Dr. Hiebert (University of Florida). For RNA extraction, purified brome mosaic virus virion was mixed with 10% SDS to a final concentration of 2%. The mixture was extracted with equal volume of phenol-chloroform twice. The aqueous phase was precipitated with ethanol and salt. The RNA was dissolved in RNase-free water and quantitated on a spectrophotometer. The purity of the extracted RNA was assessed by electrophoresis on a 1.2% formaldehyde-agarose gel.

Methyltransferase Assay

Ribose methyltransferase activity was assayed by measuring the transfer of methyl groups to the cap 0 structures at the mRNA 5' end. Reactions (50 *u*l) contained 5 *u*g brome mosaic virus RNA, 25 mM HEPES/NaOH, 1 mM DTT, and 1 *u*M Ado[Methyl-^{3}H]Met (15 Ci/mmol, Amersham) and the indicated amounts of J3R protein. After various time points during incubation at 37^{0}C, 50 *u*l samples were transferred to 50 *u*l of 4% SDS. The total 100 *u*l reaction mixture was spotted onto DEAE-cellulose filters and the filters were washed with 5% sodium phosphate four times, once with water and twice with ethanol to remove the unincorporated Ado[methyl-^{3}H]Met. Incorporation of methyl groups into RNA was determined by scintillation counting.

RNA Primer

The RNA primer, which corresponds to the 3' terminal 34-nt of a polyadenylated vaccinia virus early gene VGF transcript before polyadenylation, is 5' AUAUUUAUAAAAAUGCUAAGUAUGCGAUGUAUCU 3'. The primer was synthesized chemically and purified by HPLC (Cruchem Inc., Dulles, VA). The RNA primer was labeled at 5' end using T4 polynucleotide kinase and [γ^{32}P] ATP in a reaction containing 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 10 mM $MgCl_2$, and 100 *u*M ATP. The reaction was incubated at 37^0C for 30 min, followed by an incubation at 65^0C for 15 min.

***In vitro* Polyadenylation Assay**

The poly (A) polymerase activity was assayed by measuring the incorporation of adenylates from ATP to the 3' end of the 5' end-labeled RNA primer. Five microliters of 5' phosphorylated primer reaction mix (above) was diluted into a 50 *u*l reaction mixture containing 50 mM Tris-HCl (pH 9.0), 4.5 mM DTT, 60 mM NaCl and 1 mM ATP. Each reaction contained 100 nM of RNA primer, 27.4 nM E1L protein and the ratio of J3R/E1L was varied. After various time points post incubation at 37^0C, 2 *u*l samples were transferred to 2 *u*l of formamide containing 0.1% (vol/vol) SDS and 2.5 mM EDTA. The total 4 *u*l reaction was resolved on 12% polyacrylamide / 8 M urea gels in TBE. The gel was fixed in 10% acetic acid, dried, and then autoradiographed.

RNA Isolation

RNA was extracted from infected cells and purified by RNeasy Total RNA purification columns. Briefly, confluent BSC40 or KO3 cells ($1x10^7$ cells in 100 mm diameter dishes) was infected with wt or mutant virus at an MOI of 15 in the absence (nonpermissive condition) or in the presence of (permissive condition) IBT. At various times after infection, total cellular RNA was purified using RNeasy Total RNA purification columns as described previously (Quiagen, Inc., Chatsworth, CA). The RNA was eluted from column with DEPC-treated H_2O and quantitated by measuring its absorbance at 260 nm.

mRNA 3' End Labeling and Poly (A) Measurement

Total RNA isolated from BSC40 or KO3 cells infected with wt or mutant viruses was labeled at the 3' end in a 30 *u*l reaction on ice for 12-16 hrs. The reaction contained 20 *u*Ci [^{32}P]pCp (cytidine-3',5'-bis-phosphate, ICN), 50 mM HEPES, pH 8.3, 10 mM $MgCl_2$, 3.3 mM DTT, 10% DMSO, 10 ug acetylated BSA, 50 *u*M ATP, 10 U RNasin and 20 U T4 RNA ligase. Labeled RNA was digested with 4 *u*g of RNase A, 80 U RNase T1 in a 80 *u*l reaction containing 10 mM Tris HCl, pH 7.4, 300 mM NaCl, and 30 *u*g tRNA at 37^0C for 2 hrs. The reaction was stopped by adding 20 *u*l of stop buffer (130 mM EDTA, 2.5% SDS) and extracted with equal volume of phenol/chloroform. RNA was precipitated with 2.5 volumes of ETOH and dissolved in RNase-free water. Labeled

RNA products were analyzed by electrophoresis on 8% polyacrylamide urea gels in TBE. Gels were fixed in 10% acetic acid, dried, and then autoradiographed.

Results

Construction and Purification of His-tagged Mutant J3R Protein

Wild-type and mutant J3R proteins were overexpressed in bacteria for purification and further functional characterization of the proteins. The J3R mutant clone, PET32a-J3RG96D, was constructed by PCR amplification of the J3R coding sequence from r51x4 genomic viral DNA. It contains the precise coding region of mutant vaccinia virus gene J3R, cloned in an expression vector PET32a. The 3' primer used in PCR amplification contains a mismatch such that the PCR amplification corrects the mutation at codon 327 of gene J3R in r51x4 to the wild-type sequence. The sequence of the insert was confirmed by DNA sequence analysis. Overexpression of this clone in bacteria generated a protein with an amino terminal polyhistidine tag and a thioredoxin tag. The histidine tag is required for purification through a Ni^{++} affinity column, and thioredoxin is important for protein folding and solubility. The wt E1L, J3R and the mutant J3R proteins were overexpressed in bacteria and purified using Ni^{++} affinity chromatography. The mutant and wt J3R proteins were further purified by chromatography on a Q-sepharose column. The purity of these proteins was assayed by fractionation on the SDS-PAGE and Coomassie blue staining (Fig. 5-1). The wt and mutant thioredoxin and polyhistidine tagged J3R proteins are about 52 kD (lanes 1 and 2). The tagged E1L

protein is about 68 kD. The identity of these proteins was confirmed by reacting with E1L and J3R antisera, respectively, in western blot analysis (data not shown). The J3R and wt and mutant proteins were purified to near homogeneity. The E1L protein was not further purified due to the low yield. These proteins were shown to be functional in the biochemical assays as described below.

Biochemical Functional Analysis of J3R Mutant Protein

A standard method (188) was used to test if the mutation in gene J3R would affect the nucleoside-2'-O-methyltransferase activity. Brome mosaic virus RNA, which contains a $m^7G(5')pppG$ sequence (cap 0 structure) at the 5' end, was used as an acceptor in the methyltransferase assay with Ado[methyl-^{3}H]Met as a donor. After incubation with the J3R enzyme, the reaction mixture was spotted onto DEAE-cellulose filters and the filters were washed to remove the unincorporated Ado[methyl-^{3}H]Met. Incorporation of methyl groups into RNA was determined by scintillation counting. Three concentrations of J3R protein (16 *u*g/ml, 4 *u*g/ml and 1 *u*g/ml) were assayed in this experiment in a time course from time 0 to 60 minutes (Fig. 5-2A). The amount of methyl group incorporated into the RNA increases as the time increases and also as the amount of wt J3R protein increases (lane wt J3). The specific activity of the wt J3R protein is about 560 pmol per mg of protein per minute (Fig. 5-3B). This compares favorably with the specific activity of the wt J3R protein purified from virions after DEAE-cellulose and CM-sephadex columns, about 930 pmol per mg per minute (188). In the presence of the mutant J3R protein (Fig. 5-3A, lane mt J3), methyl incorporation

was similar to the control reaction lacking enzyme (Fig. 5-3A, lane substrate). In conclusion, the J3R G96D mutation abolishes the methyltransferase activity of the enzyme. This suggests that the ribose-2'-O-methyltransferase activity is important for the suppression of the A18R mutation.

An *in vitro* polyadenylation assay (96) was used to characterize the poly (A) polymerase processivity activity of the J3R mutant protein. Previous studies showed that E1L protein (VP55) which carries PAP activity catalyzed the addition of 30-35 adenylates to an RNA primer in a rapid highly processive burst. This initial burst was followed by a processive adenylation of additional primer molecules and a slow nonprocessive addition of adenylates to poly (A) tails that are longer than 30 nucleotides. J3R protein (VP39) dramatically stimulated the rate of elongation of the RNA primer possessing poly (A) tails that are greater than 30 nucleotides in length. The stimulatory activity of J3R was measured by the length of polyadenylates transferred from ATP to the 3' end of an RNA primer. In the assay, the RNA primer was 5'-end labeled, followed by incubation with E1L and J3R proteins in the presence of ATP in a time course (Fig. 5-3). At time 0, the 34 nt-unpolyadenylated RNA primer migrated as a discrete band (lane 1). As the time increases from time 0 to 10 min, the length of oligoadenylated products added to the RNA primer with the wt protein increases from about 34-nt to 150-nt (lanes 2 to 7). At later times, the addition of oligoadenylates by the enzymes plateaus (lanes 8 and 9). The reactions with the mutant J3R protein show a similar profile as with the wt proteins (lanes 10 to 18). The polyadenylation stimulatory activity of the mutant protein was further confirmed in the enzyme titration assay with the incubation time of 5 minutes

(Fig. 5-4). In the absence of enzymes, 34-nt unpolyadenylated VGF RNA primer migrated as a discrete band near the bottom of the gel (lane 1). In the presence of E1L protein alone, the short 55-nt oligoadenylated products characteristic of monomeric E1L protein were detected (lane 2). When both E1L and J3R proteins were in the reaction, longer oligoadenylated products were observed (lanes 3 to 8). The lengths of polyadenylated products continued to increase with increasing J3R/E1L molar ratios. The formation of the oligoadenylated products with the mutant J3R proteins is similar to that with the wt proteins (lanes 9 to 14). In conclusion, the J3R G96D mutation does not affect the polyadenylation processivity function in this *in vitro* assay. It shows that the polyadenylation processivity activity of J3R protein is not important for suppression of A18R mutation.

Length Measurement of Poly (A) Tails in wt- and mt-Infected Cells

In order to determine the effects of the J3R mutation on viral RNA metabolism, especially on 3' end formation *in vivo*, the polyadenylation status of RNAs synthesized during viral infections was analyzed. Total RNA extracted from virus-infected cells was labeled at the 3'-end with [^{32}P]pCp (cytidine-3',5'-bis-phosphate) and digested with RNase A and RNase T1. The RNase A and RNase T1 resistant poly (A) tails were analyzed by polyacrylamide gel electrophoresis and auto-radiographed. To demonstrate that total RNA isolated from infected cells at late times represents viral RNA, a pilot experiment was done with RNA isolated from wt- or J3D-infected BSC40 cells in the absence of the drug IBT (nonpermissive condition for J3D virus growth) (Fig 5-5). The

J3D mutant virus was isolated initially as an IBT-dependent virus. It contains a frameshift mutation in gene J3R and theoretically will yield a truncated J3R protein with only 58 amino acid residues. Western blot analysis (Latner unpublished) has confirmed that the wt 39 kD protein is absent from the J3D infected cell lysate. Gershon et al. have shown that deletion of the C-terminal 112 amino acids results in the loss of polyadenylation activity *in vitro* (254), therefore, we predict that the polyadenylation stimulatory function of J3R protein will be abolished in J3D. We expect that the poly (A) tail length in the J3D-infected cells will be shorter than that in wt-infected cells, which can serve as a positive control. Published results have also shown that in vaccinia virus infected mouse L cells, cellular RNA is degraded to less than 50% of its original concentration within 3 hr post infection, and it is almost totally degraded within 8 hr post infection (239). Therefore, RNAs isolated at early times (3 hr) post infection should contain both the cellular and viral RNAs while RNAs isolated at late times post infection (8 hr, 13 hr, 18 hr and 24 hr) should contain exclusively viral RNAs. Poly(A) tail analysis using RNA isolated from mock infected cells revealed cellular RNA with a poly (A) tail of about 150-nt in length (Fig. 5-5, lane 1). RNAs isolated at an early time (3 hr) following both wt and J3D infection have a similar poly (A) tail length (lanes 2 and 7), although the amount is a little diminished in J3D infection. The length of poly (A) tail of RNA isolated from wt infection at late times is about 150-200 nt (lanes 3-6), whereas J3D RNA has a short poly (A) tail of about 70-nt (lanes 8 to 11). This pilot experiment indicates that the J3D virus is incapable of making long poly (A) tails and total RNA

isolated from infected cells at late times contain exclusively viral RNA which can be used for our assay.

Using the wt and J3D virus as controls, we analyzed the poly (A) tail length of four viruses: the J3X virus, which contains the G96D mutation in gene J3R (Chapter 4), the r51x4 virus which contains both the G96D mutation, and the J3/J4 mutation and the IBT-dependent virus G2A (Fig. 5-6). Total RNAs isolated from wt or mutant infected BSC40 cells in the absence of drug IBT at 18 hr post infection were used in the 3' end labeling experiment. The poly(A) tail of wt infected cells RNA is about 150-nt long, whereas that of J3D infected cells is about 70-nt long (lanes 2 and 3), consistent with the results shown above (Fig. 5-5). The poly (A) tail length of RNA isolated from the J3X, r51x4 and G2A mutants is similar to that of wt virus (lanes 4 to 6). In conclusion, G2A, J3X and r51x4 all contain poly (A) tail of 150-200 nt long similar to that of wt.

The poly (A) length of RNA isolated from C*ts*23 infected BSC40 cells at 40^0C could not be determined due to the RNA degradation (Chapter 2). However, RNA degradation is not triggered in the RNase L knockout cell line (KO3) with a C*ts*23 infection (Chapter 2). We, therefore, performed a control end labeling assay with RNA isolated from wt and J3D-infected KO3 cells (Fig. 5-7). The quantity of long poly (A) tails observed during J3D infection is decreased compared to a wt infection (lanes 2 to 4 and lanes 5 to 7), but it is not as diminished as detected in BSC40 cells. We assume that cellular RNA degradation is slower in KO3 cells such that some residual cellular RNA can still be detected at late times post infection. Nevertheless, the results revealed a difference of long poly (A) formation in wt and J3D infected cells. We then analyzed

the poly (A) length of C*ts*23 infected KO3 cells and found that the length is normal as that of a wt virus (Fig. 5-8), although the amount is reduced consistent with reduction of mRNA.

In an attempt to test whether IBT has any effect on polyadenylation, RNAs isolated from J3D and r51x4 virus infected BSC40 cells in the presence of IBT were examined for poly (A) tail length (Fig 5-9). At an early time (2 hr) post infection, RNAs isolated from either J3D or r51x4 virus in the presence or in the absence of IBT contain both cellular and viral RNAs, and the lengths of poly (A) tail of these RNAs are the same. At late time (10 hr) post infection, the lengths of poly (A) tail of a r51x4 virus in the presence and absence of the IBT are the same, and similar to that of RNAs isolated at early time post infection. The poly (A) tail length of J3D RNAs isolated in the presence and absence of IBT are both short. Therefore, IBT has no effect on polyadenylation.

Discussion

As mentioned in Chapter 4, the J3R gene, in addition to G2R, serves as a second site suppressor of gene A18R and the point mutation at codon 96 of gene J3R is critical for this suppression. The J3R protein contains 333 amino acids. It has both the nucleoside-2'-O-methyltransferase activity and the adenylyltransferase processivity activity that are specific for the 5' end and the 3' end formation of mRNA, respectively. The experiments described here were designed to identify which function of J3R protein,

the methyltransferase activity or the adenylyltransferase processivity, is more critical for suppression of A18R *ts* mutation.

It is tempting to anticipate the involvement of the adenylyltransferase processivity of J3R in suppression of an A18R mutation due to its effect on mRNA 3' end formation. As discussed in Chapter 1, transcription termination and polyadenylation are coupled and affect the 3' end of mRNA. The mutation of the poly (A) sites and the polyadenylation factors may affect the efficiency of termination (27). Since the J3R protein is the processivity subunit of poly (A) polymerase, one might predict that mutation in the J3R gene could affect the polyadenylation function and, therefore, could shorten the length of RNA transcripts - a result opposite to the long transcripts produced by A18 mutant. However, my experiments do not provide any evidence in support of this predication. Instead, the J3R G96D mutation, which suppresses the transcription termination effect of A18R, does not affect the polyadenylation processivity function. This mutant J3R protein retains the polyadenylation processivity function in an *in vitro* assay, and the J3X virus containing the G96D mutation is capable of making long poly (A) tails in an *in vivo* assay.

In the course of characterization of the poly (A) tail formation of J3R mutant virus, we found that one IBT-dependent J3R mutant virus, J3D, contains only the N-terminal 58 amino acids of J3R. J3D makes short poly (A) tails due to the lack of the adenylyltransferase processivity function. This result is consistent with the previous *in vitro* data showing that the deletion of C-terminal 112 amino acids abolished the polyadenylation processivity function of J3R protein (254). In addition, J3D makes short

poly (A) tails even in the presence of drug IBT, a permissive condition for the mutant virus growth, suggesting that IBT has no effect on polyadenylation. In addition, J3D virus can grow in the presence of IBT with a short poly (A) tail, indicating that long 3' poly (A) is not absolutely required for virus growth. However, efforts by Gershon and coworkers to isolate a mutant vaccinia virus with a disrupted VP39 gene have failed, implying that the vaccinia J3R gene is an essential gene. Similarly, the yeast PAB1 gene has been found to be essential for cell viability (2). Lastly, both A18R *ts* mutant C*ts*23 and IBT-dependent G2A mutant contain normal poly (A) tails, suggesting that A18R and G2R protein do not affect polyadenylation.

It is interesting to note that the J3X and J3D viruses both produce shorter intermediate and late transcripts (Latner unpublished), while they show different 3' polyadenylation profiles. This suggests that the polyadenylation function of J3R may not affect the length of RNA transcripts, further supporting the notion that polyadenylation is not important in suppression of an A18R *ts* mutation.

Our methyltransferase assays show that the absence of the ribose methyltransferase function of J3R is important for the suppression of an A18R *ts* mutant. Our finding that the J3R G96D mutation abolishes the ribose-2'-O-methyltransferase activity is based on an *in vitro* experiment. Whether or not J3X virus is competent for methyltransferase function *in vivo* remains to be explored by investigating the cap structures formed in virus-infected cells. Our data, supported by studies of Gershon and coworkers, make us believe that the methyltransferase activity is deficient in J3X virus. First, our data is consistent with their mutagenesis studies used to identify the regions of

J3R protein that are important for methyltransferase activity and/or polyadenylation function (254). They identified several single amino acid substitutions which abolished methyltransfer alone or both enzyme activities. Our mutant J3R G96D protein presents another example of an amino acid substitution which destroys only methyltransferase activity and not adenylyltransferase stimulatory activity. Second, our *in vitro* data is consistent with the X-ray crystal structure analysis of J3R protein (121). The structure suggests that specific contacts between J3R protein and the substrate AdoMet, notably G68 and D95 residues in J3R, are comparable to cofactor contacts observed in other methyltransferase/cofactor complexes. Thus it is likely that this G96D mutation of gene J3R isolated in our lab is in the methyltransferase catalytic domain of the J3R protein. Third, although surface regions that are required for 3' polyadenylation stimulatory function have not been characterized by crystallographic study, a surface region of VP39 that interfaces directly with poly (A) polymerase has been identified (261). The VP55-VP39 dimerization interface lies on a surface region remote from the methyltransferase function cleft. Therefore, it is unlikely that the G96 residue is in those surface regions that are required for 3' end modification.

The role of ribose methylation is not fully understood despite the universal presence of a 2'-O-methylated nucleotide in the cap 1 structure of all animal and animal virus mRNAs. The results from several laboratories are inconclusive and controversial. *In vitro* studies comparing the cap sequences of ribosome-bound and unbound vaccinia mRNAs have shown little effect of the 2'-O-methylation on translation or ribosome binding (202). Our protein labeling experiment (Latner unpublished) shows that the

quantities of the low molecular weight proteins made in J3X-infected cells are similar to those made in a wt infection even though this J3R mutant lacks methyltransferase activity *in vitro*. This result implies that 2'-O-methylation has no effect on facilitating translation, which is consistent with the published *in vitro* studies (202). However, a recent report from Richter and coworkers by comparing injection of mRNAs containing cap 1 and cap 0 structures into oocytes indicates that cap ribose methylation of c-mos mRNA is important for translational recruitment and for the progression of the oocyte through meiosis (148). Nevertheless, the importance of cap ribose methylation in other systems is not defined. Our J3R mutant virus may serve as a good model for further study of the importance of ribose methylation in vivo.

As described in Chapter 1, transcription termination is controlled by a highly ordered complex involving RNA polymerase, transcription termination factors and antitermination factors in both prokaryotic and eukaryotic systems. Termination is likely to occur at certain pause sites which contain T-rich stretches in the nontemplate DNA strand. Some RNA polymerases may fail to proceed through these pause sites and terminate transcription, while the other polymerases may continue to elongate or backtrack. Antitermination factors could join the transcription complex at this point and help the RNA polymerase to pass through the pause sequence and to continue transcription. Phage lambda N protein is a good example of an antitermination factor. The lambda N modifies *E.coli* RNA polymerase in such a way that transcription can efficiently proceed through termination signals. Binding of the lambda N to one surface of the RNA boxB hairpin structure upstream of the terminator via the boxB-binding

domain at the N-terminal of the protein leads to a structural change of lambda N to form an alpha helix. The binding and looping triggers the binding of lambda N to the RNA polymerase and to the core antitermination complex including NusA, NusB, NusG and ribosomal protein S10 via the binding domains to the polymerase at the 3' end and to the NusA at the center of the protein. Then the elongation complex promotes the RNA polymerase to read through the termination sites (174).

We propose that A18R and J3R proteins function to regulate transcription termination in such a way that A18R serves as a termination factor, while J3R serves as an anti-termination factor, like lambda N, in the transcriptional regulation of vaccinia virus postreplicative genes. The vaccinia genome is an AT-rich structure, containing on average one T_4 stretch every 35 nucleotides and one T_5 stretch every 200 nucleotides in each DNA strand. Late during a wt virus infection, RNA polymerase may elongate along the DNA template until encountering T-rich segments and become paused or arrested. A18R could act on polymerase at pause sites and could trigger release of RNA transcripts by binding to single strand DNA and hydrolyzing ATP. The mechanism of how A18R functions as a termination factor is not very clear, however, it has been shown that an A18 mutant extract is incapable of releasing transcripts in an immobilized template assay (Aspacher unpublished data). Given the facts that the J3R mutation suppresses a mutation in the gene A18R, and that several J3R mutants cause a reduction in the sizes of intermediate and late mRNAs (Latner unpublished data), J3R may serve as an anti-termination factor. The results presented in this chapter may provide some insights on the mechanism of the regulation. The J3R 2'-O-ribose methyltransferase binds to the cap

0 structure and carries out the methylation function at the 2'-O position of the first transcribed ribose. It is not known whether this enzyme remains bound to the cap structure while the transcript extends. I have shown here that mutation in ribose methylation of J3R protein suppresses an A18R mutant. Similar to binding of RNA boxB structure by lambda N, I propose that J3R could bind to RNA transcripts at the cap site, which in turn facilitate the loading of J3R onto the RNA polymerase and the unidentified anti-termination complex at the pause site. Therefore J3R counteracts the termination function of A18R. Mutation in the ribose methylation could abolish the binding of the J3R protein to the cap structure, which in turn may abolish the loading of the J3R to the RNA polymerase and the antitermination function. I have demonstrated that J3R suppresses A18R by methylation, although whether ribose-methylation of J3R interferes directly with the transcription termination effect of A18R is not yet known.

It would not be fair not to consider some other roles of J3R that may have in the facilitation of RNA transcription elongation. J3R could recruit the elongation factors and help the RNA polymerase to continue elongation instead of release from the ternary complex. J3R protein itself could be one of the unidentified transcription elongation factors to help RNA polymerase go through the arrest sites and pause sites. These speculations of J3R in the assembly complex need to be explored by biochemistry as well as *in vivo* studies.

Fig. 5-1. Purification of His-J3R and His-E1L proteins. The purified J3R proteins from Ni^{++} columns and the purified J3R proteins from both Ni^{++} and Q-sepharose columns were seperated on SDS-polyacrylamide gels. The gels were stained with Coomassie blue for one hour and destained in 20% methanol and 7% glacial acetic acid. Sizes are denoted at the left on KD.

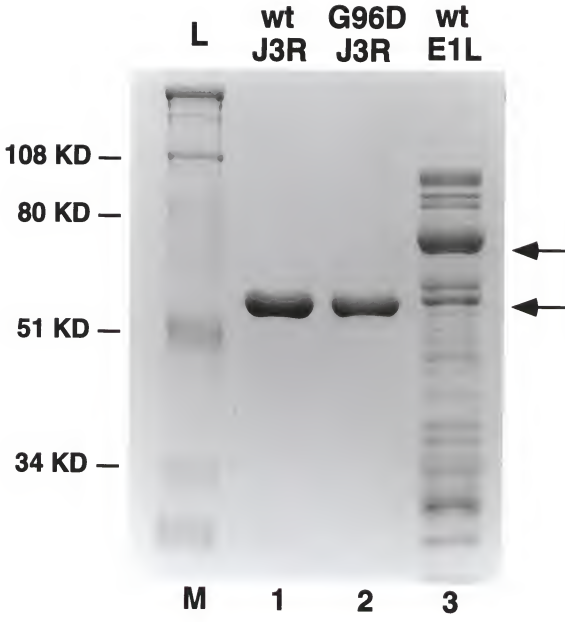
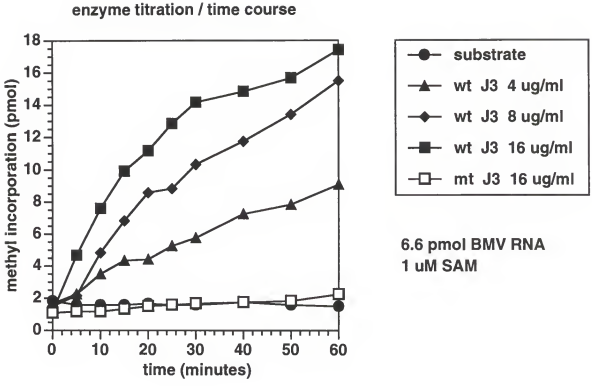
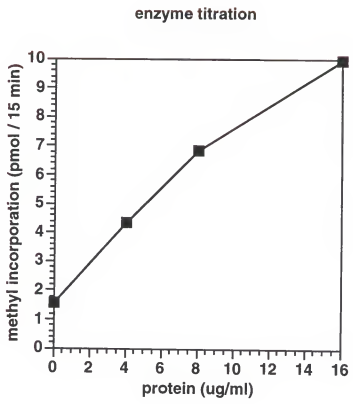

L
wt J3R
G96D J3R
wt E1L
108 KD
80 KD
51 KD
34 KD
M
1
2
3

Fig. 5-2. Methyltransferase activities of mutant J3R proteins. Reactions were done in the presence of wt or mutant J3R proteins with 4, 8, or 16 ug per ml in a time course from time 0 to 60 min. Incorporation of the methyl group from Ado[methyl-^{3}H]Met into BMV RNA as pmol was measured and plotted in the graph A. Reactions done in the absence of the enzyme were used as negative control (substrate). Specific activity of the wt and mutant protein was measured and plotted as methyl incorporation in pmol versus protein amount in ug per ml (B).

A


enzyme titration / time course
methyl incorporation (pmol)
time (minutes)
0
2
4
6
8
10
12
14
16
18
0
10
20
30
40
50
60
substrate
wt J3 4 ug/ml
wt J3 8 ug/ml
wt J3 16 ug/ml
mt J3 16 ug/ml
6.6 pmol BMV RNA
1 uM SAM

B



Specific activity = 560 pmol/min/mg

Fig. 5-3. Adenylyltransferase stimulatory activities of mutant J3R proteins in a time course assay. Purified wt and mutant J3R proteins were incubated with wt E1L protein and a 5' end labeled RNA primer in the presence of ATP. Samples were removed at the times indicated on the top of each lane in min and analyzed by 12% polyacrylamide gel and autoradiography. M, labeled 10-bp DNA ladder as size marker.

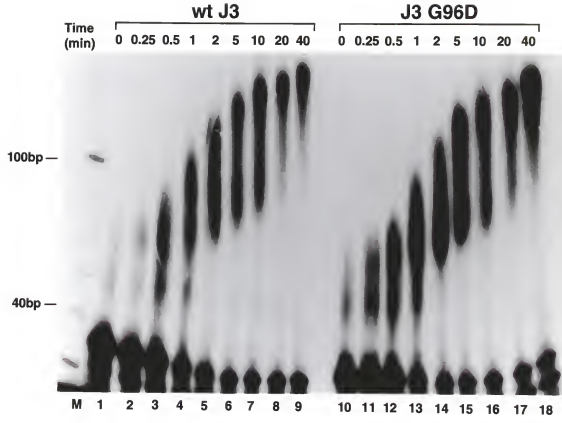

wt J3
J3 G96D
Time (min)
0 0.25 0.5 1 2 5 10 20 40
0 0.25 0.5 1 2 5 10 20 40
100bp
40bp
M 1 2 3 4 5 6 7 8 9
10 11 12 13 14 15 16 17 18

Fig. 5-4. Adenylyltransferase stimulatory activities of mutant J3R proteins in a protein titration assay. Reactions were done as described in Fig. 5-3 in the absence of E1L and J3R protein (lane 1), in the presence of E1L protein alone (lane 2) or in the presence of E1L protein and increasing amount of wt J3R protein (lanes 3 to 8) and in the presence of E1L protein and increasing amount of mutant J3R protein (lanes 9 to 14). Sizes are denoted at the left in bp of single-stranded 10-bp DNA markers.

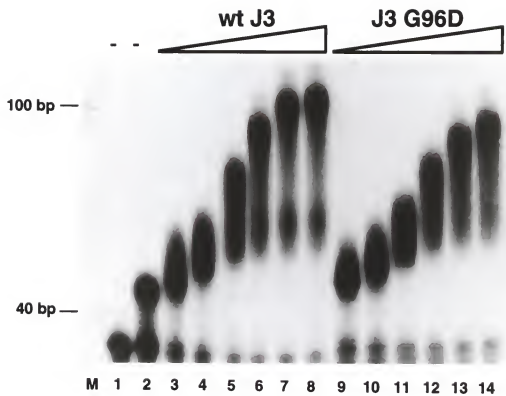

wt J3
J3 G96D
- -
100 bp
40 bp
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 5-5. Pilot experiment for poly (A) tail length measurement. BSC40 cells were infected with wt or J3D virus at an MOI of 15 and incubated at 37^0C -IBT. Total RNA was extracted from infected cells at various times post infection indicated in hr above the lanes, labeled at 3'-end with [^{32}P]pCp and digested with RNase A and RNase T1. The RNase A and RNase T_1 resistant poly (A) tails were analyzed by 8% polyacrylamide gel electrophoresis and autoradiography. M, RNA isolated from mock-infected cells. Sizes are denoted at the left in bp of single-stranded 10-bp DNA markers.

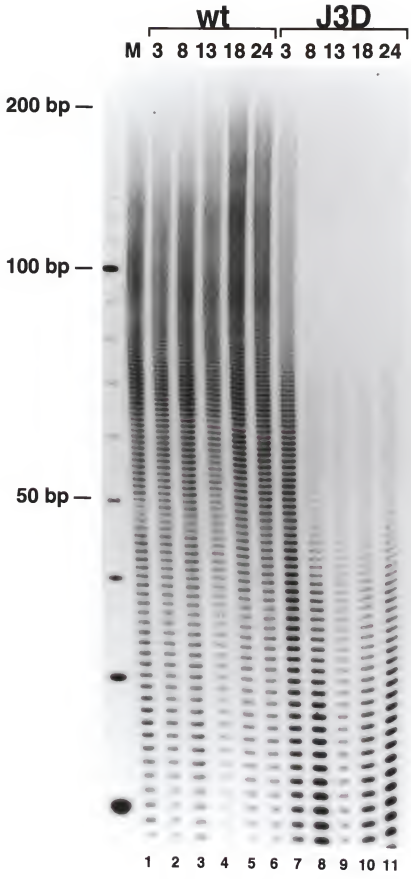

wt
J3D
M 3 8 13 18 24 3 8 13 18 24
200 bp —
100 bp —
50 bp —
1 2 3 4 5 6 7 8 9 10 11

Fig. 5-6. Length measurement of poly (A) tail of J3R and G2R mutants. RNA was isolated from wt and mutant virus-infected BSC40 cells as denoted on the top of each lane and assayed in the experiment as described in Fig. 5-5. M, mock infection. Sizes are denoted at the left in bp of single-stranded 10-bp DNA markers.

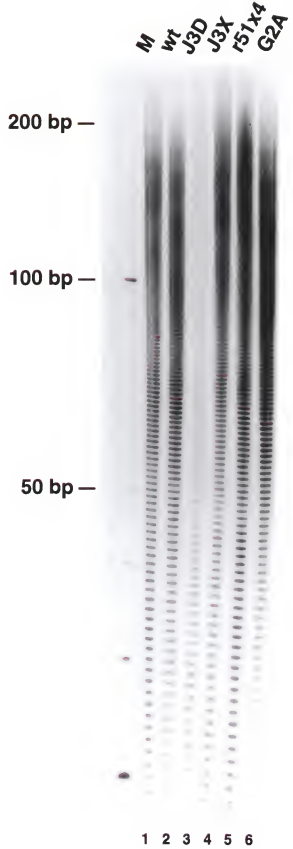

M
wt
J3D
J3X
r51x4
G2A
200 bp —
100 bp —
50 bp —
1 2 3 4 5 6

Fig. 5-7. Control experiment for poly (A) tail measurement. RNA was isolated from wt and J3D-infected KO3 cells in a time course and assayed in the experiment as described in Fig. 5-5. M, mock infection. Sizes are denoted at the left in bp of single-stranded 10-bp DNA markers.

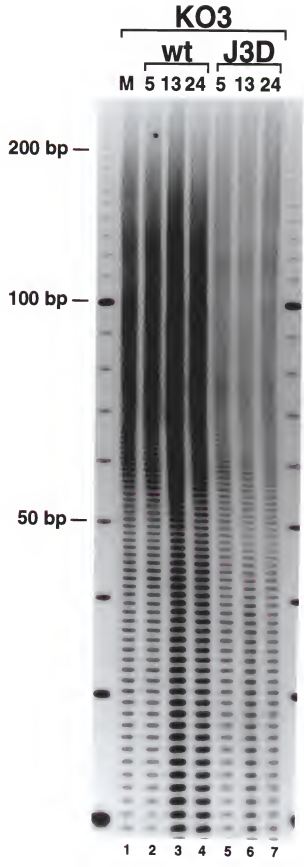
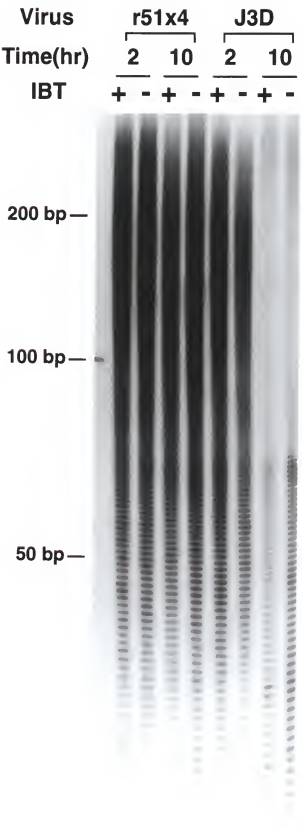

KO3
wt
J3D
M 5 13 24 5 13 24
200 bp
100 bp
50 bp
1 2 3 4 5 6 7

Fig. 5-8. Length measurement of poly (A) tail of an A18R mutant. RNA was isolated from wt and C*ts*23-infected KO3 cells as denoted on the top of each lane and assayed in the experiment as described in Fig. 5-5. M, mock infection. Sizes are denoted at the left in bp of single-stranded 10-bp DNA markers.


KO3
M
wt
J3D
ts23
200 bp —
100 bp —
50 bp —
1 2 3 4

Fig. 5-9. Length measurement of poly (A) tail of J3R mutant in the presence of IBT. RNA was isolated from r51x4 and J3D-infected BSC40 cells in the presence (+) or in the absence (-) of IBT at 2 or 10 hr post infection as denoted on the top of each lane and assayed in the experiment as described in Fig. 5-5. M, mock infection. Sizes are denoted at the left in bp of single-stranded 10-bp DNA markers.


Virus
r51x4
J3D
Time(hr)
2
10
2
10
IBT
+ - + - + - + -
200 bp
100 bp
50 bp

CHAPTER 6
GENERAL DISCUSSION AND CONCLUSION

This dissertation involves characterization the function of two vaccinia viral gene products, A18R and J3R, in transcription regulation. The early termination factors of vaccinia virus have been well defined. However, little is known of intermediate and late termination factors. The first part of my dissertation is to characterize the function of A18R in intermediate and late RNA transcription. The A18R mutant virus displays promiscuous transcription, defined as transcription within regions of the genome that are normally transcriptionally silent late during infection at non-permissive condition. Promiscuous transcription results in an increase in the intracellular concentration of double-stranded RNA, which in turn triggers activation of the cellular 2-5A pathway and subsequent RNase L-catalyzed degradation of viral and cellular RNAs. The puzzling mechanism of promiscuous transcription has haunted our laboratory for years. We hypothesized three possibilities that could account for promiscuous transcription: (i) reactivation of early promoters late during infection, (ii) random transcription initiation, (iii) readthrough transcription from upstream promoters. Previous transcriptional analysis of several viral genes argues strongly against the first two hypotheses. I tested the readthrough hypothesis in my thesis project by conducting a detailed transcriptional analysis of a region of vaccinia virus genome which contains three early genes (M1L,

M2L and K1L) positioned directly downstream of the intermediate gene, K2L. The RT-PCR results show that mutation in A18R gene results in an increased amount of readthrough transcripts of M1L gene originating from the K2L intermediate promoter. I further assessed the consequence of the A18 mutation in KO3 cells that lack RNase L. Consistently, A18R mutant infection in KO3 cells does not result in 2-5A pathway activation and results in the synthesis of longer than normal intermediate mRNAs. These data strongly suggest that promiscuous transcription results from readthrough transcription.

A18R mutant virus has been shown to be defective in late viral gene expression in KO3 cells and also in DKO cells which are defective in both the 2-5A pathway and the PKR pathway. In addition, A18R mutant virus remains temperature sensitive in both cell lines. This implies that readthrough transcription compromises downstream gene expression. Taken together, our *in vivo* characterization of A18R mutants suggests that the wt A18R gene product is a negative transcription elongation factor that may function to promote termination as I will discuss later.

My study on A18R not only solves the puzzle of promiscuous transcription and helps us to better understand intermediate and late transcription events, but also provides a model system to study transcription termination factors *in vivo*. My studies on A18R inspired the *in vitro* studies of the functions of A18R protein in transcription. Preliminary data has shown that A18R may serve as a release factor to liberate transcripts from an immobilized template driven by a vaccinia virus intermediate gene promoter (Aspacher unpublished).

Our previous in vivo analysis of an IBT-dependent G2R null mutant demonstrates that it forms intermediate and late viral mRNAs that are truncated at their 3' ends, which implies that wt G2R gene product serves as a positive transcription elongation factor of postreplicative genes. I applied genetic analysis to investigate the relationship between the A18R and G2R genes. This has shown that (1) a recombinant which contains an A18R *ts* mutation and a G2R deletion mutation was viable; and (2) three phenotypic revertants of A18R *ts* mutants contained the G2R mutations. These data imply that A18R and G2R may interact with each other directly or indirectly to regulate transcription elongation / termination.

Interestingly, I found that one phenotypic revertant of A18R *ts* mutant contained a wt G2 allele. Further characterization of the virus indicated that it was the G96D mutation in the J3R gene that suppresses A18R mutation. This implies that the J3R mutant viruses may have a defective late phenotype similar to that of the G2R mutant viruses. Further biochemical analysis has provided evidence in support of this hypothesis (Latner unpublished). Previous biochemical analysis showed that the J3R gene product is both a poly (A) polymerase subunit and an mRNA nucleoside-2'-O methyltransferase. *In vitro* biochemical analysis shows that the G96D mutation in J3R gene abolishes the ribose methylation function but not the polyadenylation processivity function. *In vivo* analysis suggests that the poly (A) formation in the J3R mutant viruses is also not affected. Therefore, the lack of the ribose methylation is important for suppression of an A18R *ts* mutation.

Based on my genetic and biochemical experiments, and previous biochemical evidence, I propose that postreplicative vaccinia transcription elongation is regulated by a dynamic complex of proteins which includes A18R, J3R, G2R, H5R, RNA polymerase, and possibly other transcription elongation and termination factors. The termination factors identified so far in both prokaryotic and eukaryotic systems can release transcripts from a ternary elongation complex, and many of them are helicases and / or nucleic acid-dependent ATPases. Previous biochemical analysis has shown that A18R gene product is also a DNA-dependent ATPase and a 3' - 5' helicase. A18R may serve as a transcription termination factor for intermediate and late gene expression. Late during a wt virus infection, RNA polymerase elongates along the DNA template until it encounters a pause site. The transcription bubble opens up and exposes the upstream near-end of the DNA strands out of the domain of the RNA polymerase. This exposure of non-template DNA strand is supported by its accessibility to nucleases and hydroxyl radicals. The exposed ssDNA may serve as an attractive site for transcription regulatory factors because *in vitro* nuclease digestion of the strand causes RNA polymerase to backtrack (305). A18R could bind to the exposed nontemplate ssDNA and physically block the backtracking of the RNA polymerase as a barricade. The 3' end of the nascent transcript which is displaced from the catalytic site of an arrested RNA polymerase may not be returned to the catalytic site of the polymerase. Therefore, transcription elongation may not resume. At the meantime, the DNA-dependent ATPase activity of the A18R gene product may hydrolyze ATP to provide necessary energy for releasing transcript from the ternary complex and therefore cause transcription termination. In the absence of the function of

A18R, the transcription termination event is avoided and significant amounts of readthrough transcripts are produced.

I propose that J3R may act in a similar way to lambda N, an antitermination factor, in joining into the elongation complex when RNA polymerase pauses, promoting the RNA polymerase to read through the pause and termination sites. The mechanism of action of J3R in transcriptional regulation may involve binding of nascent RNA transcripts at the cap structure and recruiting transcription elongation factors, such as G2R, to the elongation complex. With the cooperation of G2R, and maybe with other elongation factors, J3R may rescue the RNA polymerase from the pause and arrest sites via the effects on the backtracking of the polymerase in two different ways. It may prevent backtracking of the RNA polymerase at the pause site and promote the RNA polymerase to continue elongation. Alternatively, it may promote backtracking of the RNA polymerase at the arrested site and promote the cleavage of the displaced 3' end of the transcripts, which may in turn facilitate the polymerase to continue elongation. In the absence of the function of J3R or G2R, A18R executes its termination effect and produces shorter than normal transcripts. In vitro transcription elongation and termination assays using wt, G2R, J3R, and A18R mutant infected cell extracts will provide additional information.

In conclusion, my dissertation project establishes the role of A18R as an intermediate and late transcription termination factor and evaluates the interaction of A18R and J3R and G2R during late transcription elongation/ termination. This result provides us an *in vivo* model to study the events during intermediate and late

transcription. Because the vaccinia virus transcription apparatus is similar to these of eukaryotes, it may provide an understanding of transcription elongation/ termination of eukaryotes.

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BIOGRAPHICAL SKETCH

On April 3, 1969, Ying Xiang was born in Shanghai, a beautiful port city along Yangtse River in China. She was brought up and greatly influenced by her diligent and caring grandmother and parents. As naturally good hearted as she is, Ying wanted to be a good doctor to help people. After the fierce competition, Ying was accepted to Shanghai Medical University, the highest rated medical school in China in 1987. She struggled through the tedious medical courses and the tough drills of ward rounds until Jiong came to her side. They helped each other and gradually liked each other through six years of medical education. Her dear grandmother suffered from cervical cancer before Ying graduated from medical school, which made up her mind to become an oncologist. In 1993, she joined Cancer Hospital. She was very depressed when she saw a lot of patients passed away even after suffering tremendous radiotherapies and chemotherapies. She realized from deep down that she needed to enrich herself with basic research experience, which might help her to find cures for the disease rather than simply dealing with the symptoms from day to night.

She and Jiong decided to take a brave move to pursue their research careers in the United States. They came to Gainesville, Florida, in 1994. Ying chose to join the Department of Molecular Genetics and Microbiology under the supervision of Dr.

Richard Condit while Jiong did his Ph.D. in the Department of Pharmacodynamics. Once again, they helped each other through five years of graduate courses and research work. Now Ying will get her Ph.D. in December of 1998 and Jiong has also got his. They plan to move to Cleveland, Ohio, to continue their journey of life and science.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Richard C. Condit, Chair
Professor of Molecular Genetics and Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Sue A. Moyer
Professor of Molecular Genetics and Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

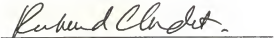
William Hauswirth
Eminent Scholar of Molecular Genetics and Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Thomas Rowe
Associate Professor of Pharmacology and Therapeutics

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 1998



Dean, College of Medicine

Dean, Graduate School